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THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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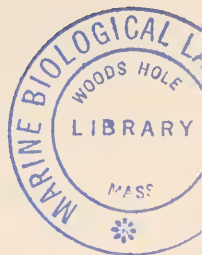
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VOLUME 131
JULY TO DECEMBER, 1966



Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, \$3.75. Subscription per volume (three issues), \$9.00.

Communications relative to manuscripts should be sent to Dr. Donald P. Costello, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P.O. Box 429, Chapel Hill, North Carolina 27514, during the remainder of the year.

Second-class postage paid at Lancaster, Pa.

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised August 15, 1963)

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees *ex officio*, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti*, who shall be elected from present or former Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that Trustees *Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected

annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected for a term of five years and shall serve until his successor is selected and qualified, except that such term shall not run beyond the Annual Meeting of the Board following his 65th birthday; candidates over the age of 65 shall be elected on an annual basis. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED AT TRUSTEE MEETING AUGUST 16, 1963—EXECUTIVE COMMITTEE

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of ten members as follows: *ex officio* members who shall be the Chairman of the Board of Trustees, President, Director and Treasurer; six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term.

(B) The President shall act as Chairman of the Executive Committee and the Chairman of the Board of Trustees as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine.

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or by Law.

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its actions shall be reported to the Board of Trustees.

II. RESOLVED:

The elected members of the Executive Committee shall be constituted as a standing "Committee for the Nominations of Officers," responsible for making nominations at the annual meeting of the Corporation and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire. (Chairman of the Board, President, Director, Treasurer, and Clerk.)

IV. REPORT OF THE DIRECTOR

TO: THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY

Gentlemen:

I submit herewith the report of the seventy-eighth session of the Marine Biological Laboratory.

1. Facilities Developments

Early in June an application was made to the National Science Foundation for a grant of \$2,200,000.00 to match the grant in the same amount contingently made to the Laboratory by the Ford Foundation. The projected plan of the Laboratory calls for the construction of a new instructional building, to replace the present outmoded wooden frame buildings, and a new dining hall-dormitory.

A Building Committee was appointed which in turn selected the firm of Peirce, Pierce, and Luykx to develop plans for the projected building. In order to meet the requests from the National Science Foundation for detailed plans, a number of meetings were held by the Building Committee with the heads of the various training programs and the architects. A number of plans were developed by the architects and finally one was tentatively adopted subject to modifications ensuing from engineering feasibility and contractors' bids.

During the course of the winter, title to Center Street was transferred from the Town of Falmouth to the Laboratory, increasing the area available for the new training building. Also, the Laboratory has acquired by purchase "Re House," the second house north of North Street with frontage on the Eel Pond, formerly the property of Mrs. Warren Vincent. The total area included is 7100 square feet.

2. Policy on Operation

During the past several years the Trustees of the Laboratory have been keenly interested in realizing the full potentialities of the Laboratory not only during the summer months but during the rest of the year. The prospect of a year-round instructional building and heated student dormitory provides opportunities for broadening the scope of the Laboratory during the non-summer months. The large number of highly qualified students who cannot be accommodated during the summer session prompted the Laboratory to formulate policy encouraging instruction during the winter months.

In line with the new policy the Marine Biological Laboratory will entertain proposals for the off-season use of its facilities by other institutions offering teaching

and research programs of a high caliber, or scientists interested in similar programs in their fields of interest. The Laboratory will sponsor applications for funds in support of such programs and will encourage participation by well qualified individuals and groups. Also, the Laboratory will continue to make its facilities available to qualified scientists who wish to conduct research or read in the library during the non-summer months.

3. Winter Operation

During recent years there has been a continuing significant increase in the use of the facilities during the non-summer months. This current winter (1965-1966) there have been 31 investigators in residence at the Laboratory, supplemented by a technical staff of the same number (31) occupying 42 laboratories. In addition there have been 30 investigators at the Laboratory on a transient basis to read in the library, collect certain seasonal biological materials, or for limited research activity.

The services at the Laboratory are maintained during the non-summer months. The Supply Department must devote considerable time in preparation for the push of the coming summer, reconditioning the boats, docks, floats, and gear, but at the same time maintaining its collecting activities for the resident investigators and for the exploratory work of the Systematics-Ecology Program. Also during this period, active collecting is maintained for the supply of live marine forms to investigators at their home institutions. During this current period over 1000 shipments of material will be made.

During the winter the library operates as it does during the summer months, twenty-four hours a day for the seven days of the week. It serves the resident and visiting investigators at the Laboratory, the more than 135 scientists at the Woods Hole Oceanographic Institution, and the scientific personnel at the Laboratory of the Bureau of Commercial Fisheries.

The Apartment House is open throughout the year, so is available to both long-term and short-term investigators and visitors at the Laboratory during the non-summer months, as well as visiting investigators at the Woods Hole Oceanographic Institution.

4. Systematics-Ecology Program

Plans have gone forward for the new Laboratory survey boat for this program. The initial Ford Foundation Grant of \$100,000.00 has been supplemented by additional funds from the Grass Foundation (\$10,000.00) to complete the outfitting of the boat. A generous donation of some surplus equipment by the Oceanographic Institution was most welcome.

The scuba diving program has been expanded and now includes personnel in both the Systematics-Ecology Program and in the Supply Department.

5. Committee on Organization

A Committee on Organization was appointed at the end of the summer (1965), under the Chairmanship of Dr. Eric G. Ball, to scrutinize the present administrative set-up of the Laboratory with a view to making recommendations on modifications

which will specify the responsibilities of the various officers in relation to the Executive Committee and the Board of Trustees. The Committee was also charged to look into the advisability of including on the Board of Trustees a limited number of "lay" trustees, men of public affairs interested in the Laboratory and its operation. It is anticipated that the Committee will submit its final report early in the summer (1966).

6. Deaths

This past year the Laboratory suffered irreparable losses by death: Dr. Arthur K. Parpart, President of the Corporation, who first came to the Laboratory as a graduate student and who, in his mature years, has played a prominent and constructive role in the development of the Laboratory; Mr. James H. Wickersham, Treasurer of the Corporation, who gave generously of his time and talents in so effectively managing the financial interests of the Laboratory; and Dr. Ethel B. Harvey, renowned for her investigations into the embryology of *Arbacia*, who probably had worked more summers continuously in annual residence at the Laboratory (since 1907) than any other investigator in its history.

7. Personnel Changes

At the mid-winter meeting of the Trustees on February 12, 1965, Dr. Armstrong was elected to continue as Director of the Laboratory until the August meeting of the Trustees in 1966.

Dr. H. Burr Steinbach was invited to accept the Directorship of the Laboratory at the retirement of Dr. Armstrong, which he has consented to do. Dr. Steinbach will bring to the office an intimate knowledge of the history, traditions and operations of the Laboratory growing out of an association of over thirty years with the institution. Also, he has had a broad experience in the administration of scientific organizations. At the mid-winter meeting of the Trustees on February 18, 1966, Dr. Armstrong was elected President of the Corporation to complete the unexpired term of Dr. Parpart, and Mr. Alexander T. Daignault was elected Treasurer of the Corporation to complete the unexpired term of Mr. James H. Wickersham. Mr. Gerard Swope, Jr. expressed a desire to retire as Chairman of the Board, but was prevailed on to continue to serve until the summer of 1966.

Two promotions have been made in recognition of meritorious service to the Laboratory. Miss Jane Fessenden was advanced from Acting Librarian to Librarian and Mr. John J. Valois was appointed Assistant Manager of the Supply Department.

1. MEMORIALS

ERNST ALBERT SCHARRER

BY ARNOLD LAZAROW

The life of Dr. Ernst Albert Scharrer, a leading neuroendocrinologist and head of the Department of Anatomy at the Albert Einstein College of Medicine, in New York, came to an untimely end on April 29th at Sarasota, Florida, in a drowning accident.

Dr. Scharrer was born in Germany on August 1, 1905. He was educated at the

University of Munich where he earned his Ph.D. in zoology in 1927, and his medical degree in 1933. Having served as a Sterling Fellow at Yale University from 1929 to 1930, and as an assistant in zoology at the University of Vienna from 1930 to 1931, he was appointed an investigator at the Research Institute for Psychiatry in Munich in 1931. Two years later, he was put in charge of the Neurological Institute at the University of Frankfurt-am-Main where he remained until 1937.

At this time, however, Dr. Scharrer became very concerned about the changing political events in his native country and he was worried about the consequences of Hitler's rise to power. Although the Scharrers were not members of the minority group, and could have remained in Nazi Germany without fear of persecution, they refused to become passive participants in the horrible inhumanities that were to follow. Knowing intuitively what was to come, Ernst and Berta Scharrer decided to leave Germany. Receiving a Rockefeller Foundation fellowship, he served as a visiting scientist at the University of Chicago during 1937 and 1938 and at the Rockefeller Institute in New York for the next two years. In 1940 he accepted a position as Assistant Professor of Anatomy at Western Reserve University in Cleveland. After six years there he moved to the University of Colorado where he served as Associate Professor until 1954. In 1955 he was appointed Professor and Chairman of Anatomy at the newly created Albert Einstein College of Medicine. Included among his many outside activities was a term of service on the Morphology and Genetics Study Section of the U. S. Public Health Service from 1954 to 1959.

Dr. Scharrer spent his first summer at the Marine Biological Laboratory, Woods Hole, in 1937, and he returned to this Laboratory during a dozen subsequent summers, collecting material and working as a research investigator. He was elected a Trustee of the Laboratory in 1962 and he is included among the list of distinguished Friday Evening Lecturers.

As a teacher and a lecturer, Dr. Scharrer was unexcelled. His capacity for clear presentation, his superb drawing ability (being able to create the most intricate three-dimensional blackboard illustrations without the use of a single note), his clever selection of examples and use of analogies, his great enthusiasm for the subject matter and his exquisite sense of drama all contributed to make each of his lectures a finished theatrical performance as well as an artistic and educational experience.

Dr. Scharrer has made important and imaginative research contributions to the field of neuroendocrinology and to our knowledge of the microscopic vascular architecture of the brain. In one of his earliest papers published in 1928 Scharrer described the presence of secretory droplets within specialized nerve cells in the thalamus of *Fundulus heteroclitus*. Similar cells had been described earlier in the spinal cord of the skate by Speidel (1919). On the basis of his faith in morphologic observations, Scharrer suggested that these nerve cells have the capacity to secrete a hormonal substance. However, the concept of neurosecretion was not quickly accepted and during the following decades Dr. Scharrer vigorously defended the thesis of neurosecretion while he continually extended his observations and accumulated more and more convincing evidence. Radical new ideas are slow to gain acceptance and most biologists were reluctant to believe that nerve cells had a secretory function. In their *Physiological Reviews* article entitled "Neurosecretion" and published in 1945, the Scharrers summarized the then-current evidence for neurosecretion; however, even at that time they were still "lone voices in the wilderness." But in the ensuing years physiological and biochemical studies by other investigators provided strong supporting evidence for neurosecretion and as time passed this concept was challenged less and less. At present, the idea of neurosecretion is not only widely accepted, but has become a most fashionable field of research endeavor. During the last decade there have been increasing numbers of conferences, symposia, and monographs devoted to this area.

In a review paper published in the *American Scientist* in 1951 Bargmann and Scharrer established the thesis that the antidiuretic, oxytocic and vasopressor hormones are synthesized in the supraoptic and paraventricular nuclei of the hypothalamus rather than in the posterior lobe of the pituitary, as had been generally believed. These hormones are then transported in the form of particulate droplets within and along the axons, by way of the hypothalamo-hypophyseal tract to the neurohypophysis where they are stored and ultimately released into the blood.

Subsequent studies by many investigators have now established the role of the hypothalamus in controlling the release of adrenocorticotropin, gonadotropins, thyrotropin and growth hormone. Specific neurohormones (releasing factors) are synthesized in the hypothalamus; they are presumably transported along axons to the median eminence where they enter the pituitary-portal circulation. Upon reaching the adenohypophysis they serve, for example, as the adrenocorticotropin or thyrotropin releasing factors. Thus, the concept of neurosecretion now relates directly or indirectly to almost all phases of endocrine function.

It should be emphasized that at the time of their initial studies, the Scharrers were already aware of the broad phylogenetic significance of the concept of neurosecretion, having demonstrated that neurosecretory cells and neurosecretory tracts were widely distributed throughout the animal kingdom. Dr. Scharrer used marine material extensively in his investigations for he was aware that marine forms often provided specialized features that were uniquely adapted to the solution of a particular problem.

In all, Dr. Scharrer was the author or co-author of 92 publications and these included a multiplicity of original articles, many reviews, and a book entitled *Neuroendocrinology*, published by the Columbia University Press (1963). Nine of these publications, including the book on neuroendocrinology, were written jointly with his wife Berta; others were written with collaborators including Wolfgang Bargmann, Sanford L. Palay and others.

In all of his scientific work, Dr. Scharrer enjoyed a very close association with his wife Berta. They met in Munich and they were married in 1934. They were constantly together, both at work and at play. They formed a team in which they completely complemented each other, and yet each pursued an independent area of research. For a time, they divided the animal kingdom between them, with Ernst Scharrer concentrating on vertebrates and Berta Scharrer studying invertebrates. It is difficult to think of Ernst without also thinking of Berta.

It is a great tribute to Ernst Scharrer and to Berta's strength of character that on the Monday which followed the tragic Thursday in Florida, Berta returned to the Department of Anatomy at Einstein and, despite her great personal grief, assumed the responsibility of serving as acting Department head. Were he here, Ernst would be very proud of the way Berta has carried out this responsibility and in the way the Department has responded.

I would like to say a few words about Ernst Scharrer—the man. He was an individual with great moral strength and the highest ethical principles. He was willing to speak out against the things he thought wrong and to vigorously defend the things he believed in. Dr. Scharrer had extremely high standards for everything he did; he demanded near perfection of himself and urged his students and associates to emulate these high standards. Yet he was sympathetic and understanding of the problems of others. He had a love of life and a zest for hard work. His wonderful sense of humor always enlivened the discussion of any group. Ernst Scharrer was one of the rarest of men for he was a superb teacher, an imaginative and productive research investigator, and a truly delightful man. He was a devoted husband to Berta and a very warm personal friend to many of us here at Woods Hole.

2. THE STAFF

EMBRYOLOGY

I. INSTRUCTORS

JAMES D. EBERT, Director, Department of Embryology, Carnegie Institution of Washington, in charge of course

DONALD D. BROWN, Staff Member, Department of Embryology, Carnegie Institution of Washington

ALLISON L. BURNETT, Associate Professor of Biology, Western Reserve University

ROBERT L. DEHAAN, Staff Member, Department of Embryology, Carnegie Institution of Washington

TOM HUMPHREYS, Assistant Professor of Biology, Massachusetts Institute of Technology

THOMAS J. KING, Head, Department of Embryology, Institute for Cancer Research, Philadelphia

JAMES W. LASH, Associate Professor of Anatomy, University of Pennsylvania

II. JUNIOR INSTRUCTOR

SIDNEY B. SIMPSON, Department of Anatomy, Western Reserve University

III. LECTURER

HEWSON SWIFT, Professor of Zoology, University of Chicago

IV. LABORATORY ASSISTANTS

C. B. KIMMEL, The Johns Hopkins University

DAVID E. KOHNE, Purdue University

V. LECTURES

J. D. EBERT	Perspectives in developmental biology
T. J. KING	Analysis of early teleost development
T. J. KING	Analysis of developmental processes in teleosts
JANE OPPENHEIMER	Differentiation of the lens in <i>Fundulus</i>
HANS LAUFER	Nucleocytoplasmic interactions during insect development
W. H. TELFER	Formation of yolk—the relative roles of ovarian synthesis and protein uptake
E. ANDERSON	Some aspects of the fine structure of oocyte differentiation and vitellogenesis in the roach, <i>Periplaneta americana</i>
R. A. WALLACE	Biochemical aspects of vertebrate yolk formation and structure
A. C. BLACKLER	Experiments with amphibian embryonic sex cells
TOM HUMPHREYS	The regeneration of sponges from dissociated cells
TOM HUMPHREYS	The molecular basis of species-specific cell sorting in sponges
M. S. STEINBERG	Cellular mechanisms in tissue reconstruction
M. S. STEINBERG	The cell surface in morphogenesis
R. L. DEHAAN	Cell contact interactions: Organogenesis
H. COON	Clonal stability of a differential phenotype: or, chondrocyte dedifferentiation revisited

J. W. SAUNDERS, JR.	Epithelial-mesenchymal interactions in limb development
A. BURNETT	Growth polarity and form regulation in hydroids
A. BURNETT	Pathways of cellular differentiation in hydroids
A. BURNETT	A model of cell differentiation in hydroids
R. FLICKINGER	Regeneration in planaria
C. S. THORNTON	Some epidermal and neural factors in limb regeneration in larval salamanders
ELIZABETH HAY	Fine structure of regenerating limbs
R. J. GOSS	Compensatory hypertrophy
J. W. LASH	Ascidians I
J. W. LASH	Ascidians II
J. W. LASH	Ascidians III
HEWSON SWIFT	Cytochemical studies of nucleocytoplasmic interactions
HEWSON SWIFT	Nucleic acids in mitochondria and chloroplasts
HARRY EAGLE	Biochemical consequences of cellular interaction in culture
J. PAPACONSTANTINOU	Protein and nucleic acid changes in the differentiation of lens cells
ARON MOSCONA	Changes in glutamine synthetase activity in the neural retina of the chick embryo <i>in situ</i> and <i>in vitro</i>
R. L. DEHAAN	Annelids, molluscs, echinoderms I
R. L. DEHAAN	Annelids, molluscs, echinoderms II
DAVID EPE	Early biochemical reactions of fertilization
D. D. BROWN	Biochemistry of oogenesis, fertilization and early development I
D. D. BROWN	Biochemistry of oogenesis, fertilization and early development II
JOHN GURDON	Nuclear transplantation and the control of gene activity
T. J. KING	Developmental capacity of nuclei of frog renal adenocarcinoma cells
D. D. BROWN	Biochemical consequences of nuclear transplantation
J. F. ALBRIGHT	Competence of cells for antibody formation
A. SILVERSTEIN	The development of immunity in the mammalian fetus
B. F. ARGYRIS	Immunological tolerance
LAURENS RUBEN	Post-embryonic cell differentiation: normal and neoplastic
LAURENS RUBEN	Post-embryonic induction in urodele limbs
ROBERT GOOD	Ontogeny and phylogeny of the immune response
*S. GELFANT	The cell division cycle
*Y. BERWALD	Chemical carcinogenesis <i>in vitro</i>
*S. SIMPSON	Aspects of appendage regeneration in lizards
*L. SAXÉN	Methods in teratology

* Post-course lectures.

PHYSIOLOGY

I. CONSULTANTS

MERKEL H. JACOBS, Professor of Physiology, University of Pennsylvania

ARTHUR K. PARPART, Professor of Biology, Princeton University

ALBERT SZENT-GYÖRGYI, Director, Institute for Muscle Research, Marine Biological Laboratory

W. D. McELROY, Director, McCollum-Pratt Institute, The Johns Hopkins University

II. INSTRUCTORS

- J. WOODLAND HASTINGS, Professor of Biochemistry, University of Illinois, in charge of course
 EDWARD A. ADELBERG, Professor of Microbiology, Yale University
 HARLYN HALVORSON, Professor of Molecular Biology, University of Wisconsin
 SHINYA INOUÉ, Professor of Cytology, Dartmouth College
 K. E. VAN HOLDE, Professor of Physical Chemistry, University of Illinois (on leave 1965)
 FRED KARUSH, Professor of Microbiology, University of Pennsylvania
 WILLIAM F. HARRINGTON, Professor of Biology, The Johns Hopkins University
 HANS KORNBERG, Professor of Biochemistry, Leicester University, England

III. SPECIAL LECTURERS

- QUENTIN H. GIBSON, Professor of Physiology, University of Pennsylvania
 ANDREW SZENT-GYÖRGYI, Professor of Cytology, Dartmouth College
 R. K. CLAYTON, C. F. Kettering Laboratory, Yellow Springs, Ohio

IV. LABORATORY ASSISTANTS

- GEORGE KISSIL, University of Connecticut
 JOHN CLARK, Dartmouth College

V. STAFF LECTURES

- | | |
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| J. WOODLAND HASTINGS | The generation and utilization of excited states in bioluminescent systems |
| J. WOODLAND HASTINGS | Bioluminescent systems: II |
| HARLYN HALVORSON | The synthesis of protein in cell-free systems |
| HARLYN HALVORSON | Ordered transcription of the genome |
| HARLYN HALVORSON | Intracellular differentiation in <i>Bacillus</i> |
| HANS KORNBERG | Integration of metabolism: I |
| HANS KORNBERG | Integration of metabolism: II |
| HANS KORNBERG | Integration of metabolism: III |
| FRED KARUSH | The specific interaction of antibody |
| FRED KARUSH | The nature of immunoglobulins |
| FRED KARUSH | Biological aspects of the immune response |
| EDWARD A. ADELBERG | The chemical basis of mutation |
| EDWARD A. ADELBERG | Effects of mutation on protein synthesis |
| EDWARD A. ADELBERG | Genetic recombination in bacteria: I |
| EDWARD A. ADELBERG | Genetic recombination in bacteria: II |
| SHINYA INOUÉ | Fine structural basis and physiology of mitosis |
| SHINYA INOUÉ | The use of polarized light for analysis of biological fine structure |
| SHINYA INOUÉ | DNA and chromosome arrangement in living sperm |
| W. F. HARRINGTON | Stereochemistry of polypeptide chains |
| W. F. HARRINGTON | Structure of fibrous proteins: I |
| W. F. HARRINGTON | Structure of fibrous proteins: II |
| QUENTIN H. GIBSON | Hemoglobin-ligand reactions |
| QUENTIN H. GIBSON | Mechanisms of some flavoprotein enzyme reactions |
| R. K. CLAYTON | Physical and photochemical mechanisms in photosynthesis |
| R. K. CLAYTON | The significance of light emitted by photosynthetic tissues |

VI. INVITED LECTURES

IRVIN ISENBERG	Electron spin resonance in biochemistry
HENRY MAHLER	Properties of flavoproteins
DANIEL MAZIA	Chemical resolution of chromosomes
C. S. VESTLING	Structural studies on lactic dehydrogenase
CYRUS LEVINTHAL	Computer construction and display of molecular models
HARRY GINSBERG	Replication of animal viruses
B. D. DAVIS	Antimicrobial agents as physiological tools
W. P. JENCKS	Effects of solvents on protein structure
TERU HAYASHI	Physiological and molecular aspects of muscle contraction
GEORGE WALD	The retinal basis of human vision
PHILIP HANDLER	Enzymatic mechanisms
MATTHEW MESELSON	Genetic recombination
DAVID HOGNESS	The structure and function of lambda DNA
R. M. BOCK	Recent developments in the study of soluble RNA
CARL WOESE	The genetic code: Is it really solved?
F. RITTOSA	The distribution of DNA complementary to ribosomal and soluble RNA
SEYMOUR COHEN	Comparative biochemistry of D-arabinose and its nucleosides
ALVIN NASON	Enzymology of inorganic nitrogen metabolism: I
ALVIN NASON	Enzymology of inorganic nitrogen metabolism: II
SOL SPIEGELMAN	Communication between a virus and host cell: A comparison of single-stranded DNA and RNA
SOL SPIEGELMAN	Problems for replication for single-stranded DNA and RNA viruses
SOL SPIEGELMAN	Synthesis of a self-propagating infectious nucleic acid with a purified enzyme
ALBERT SZENT-GYÖRGYI	Growth

MARINE BOTANY

I. CONSULTANT

WILLIAM RANDOLPH TAYLOR, Professor of Botany, University of Michigan

II. INSTRUCTORS

WALTER R. HERNDON, Professor of Botany, University of Tennessee, in charge of course

PHILIP W. COOK, Assistant Professor of Botany, University of Vermont

H. WAYNE NICHOLS, Associate Professor of Botany, Washington University

FRANK E. ROUND, Lecturer in Botany, University of Bristol, England

ROBERT T. WILCE, Assistant Professor of Botany, University of Massachusetts

III. SPECIAL LECTURERS

R. W. HOLTON, Department of Botany, University of Tennessee

I. M. LAMB, Farlow Herbarium, Harvard University

JOHN KINGSBURY, Department of Botany, Cornell University

LUIGI PROVASOLI, Haskins Laboratories, New York

FRANCIS R. TRAINOR, Department of Botany, University of Connecticut

IV. LABORATORY ASSISTANTS

RUSSELL G. RHODES, University of Tennessee

JEFFERY S. PRINCE, University of Massachusetts

V. COLLECTOR

DAVIS L. FINDLEY, University of Tennessee

VI. LECTURES

WALTER R. HERNDON	Marine plants and the plant kingdom: Introduction to the algae
PHILIP W. COOK	Chlorophyceae; Volvocales
PHILIP W. COOK	Volvocales
H. WAYNE NICHOLS	Cultivation of marine and fresh-water algae
PHILIP W. COOK	Cladophorales
H. WAYNE NICHOLS	Ulotrichales
ROBERT T. WILCE	Ulvaes
ROBERT T. WILCE	Siphonales, Siphonocladales, Dasycladales
WALTER R. HERNDON	Chlorococcales
WALTER R. HERNDON	Chlorococcales, Tetrasporales
PHILIP W. COOK	Oedogoniales
PHILIP W. COOK	Zygnematales I
PHILIP W. COOK	Zygnematales II
PHILIP W. COOK	Zygnematales III
FRANK E. ROUND	Chrysophyta, introduction Xanthophyceae
FRANK E. ROUND	Chrysophyta, Chrysophyceae I
ROBERT T. WILCE	Phaeophyta, introduction to morphology and ecology; Ectocarpates
ROBERT T. WILCE	Phaeophyta I
ROBERT T. WILCE	Phaeophyta: Chordariales
FRANK E. ROUND	Chrysophyta, Chrysophyceae II
FRANK E. ROUND	Chrysophyta, Bacillariophyceae I
FRANK E. ROUND	Chrysophyta, Bacillariophyceae II
FRANK E. ROUND	Chrysophyta, Bacillariophyceae III
WALTER R. HERNDON	Euglenophyta
ROBERT T. WILCE	Phaeophyta II
ROBERT T. WILCE	Phaeophyta III
L. PROVASOLI	Nutrition and physiology of algae
L. PROVASOLI	Algae as food for other organisms, especially invertebrates
ROBERT T. WILCE	Phaeophyta IV
ROBERT T. WILCE	Phaeophyta V
PHILIP W. COOK	Cyanophyta
H. WAYNE NICHOLS	Rhodophyta I
H. WAYNE NICHOLS	Rhodophyta II
I. M. LAMB	Sublittoral antarctic benthic algae
H. WAYNE NICHOLS	Rhodophyta III
H. WAYNE NICHOLS	Rhodophyta IV
H. WAYNE NICHOLS	Rhodophyta V
FRANCIS TRAINOR	Morphogenetic phenomena in green algae; demonstration of zoospore formation and sexual reproduction in <i>Scenedesmus</i>
H. WAYNE NICHOLS	Rhodophyta VI

H. WAYNE NICHOLS	Rhodophyta VII
WALTER R. HERNDON	Charophyta
H. WAYNE NICHOLS	Rhodophyta VIII
H. WAYNE NICHOLS	Rhodophyta IX
FRANK E. ROUND	History of marine basins
JOHN KINGSBURY	Environment of attached marine algae; toxic algae; periodicity in growth of <i>Derbesia-Halicystis</i>
PHILIP W. COOK	Pyrrophyta
ROBERT T. WILCE	Ecology of arctic algae
R. W. HOLTON	Physiology and biochemistry of blue green algae

INVERTEBRATE ZOOLOGY

I. CONSULTANTS

FRANK A. BROWN, JR., Morrison Professor of Biology, Northwestern University
 LIBBIE H. HYMAN, American Museum of Natural History
 CLARK P. READ, Professor of Biology, Rice University
 ALFRED C. REDFIELD, Woods Hole Oceanographic Institution

II. INSTRUCTORS

W. D. RUSSELL HUNTER, Professor of Zoology, Syracuse University, in charge of course
 GEORGE G. HOLZ, JR., Professor of Microbiology, State University of New York, Upstate Medical Center
 NORMAN MILLOTT, Professor of Zoology, Bedford College, University of London, England
 IRWIN W. SHERMAN, Assistant Professor of Zoology, University of California, Riverside
 ALLAHVERDI FARMANFARMAIAN, Professor of General Physiology, Pahlavi University, Shiraz, Iran
 ERIC L. MILLS, Assistant Professor of Biology, Queen's University, Kingston, Ontario, Canada
 FRANK M. FISHER, JR., Assistant Professor of Biology, Rice University
 ROBERT K. JOSEPHSON, Associate Professor of Biology, Western Reserve University

III. ASSISTANTS

JOHN H. BUSSEY, University of Rhode Island
 W. BRUCE HUNTER, University of California, Santa Barbara

IV. LECTURES

ROBERT K. JOSEPHSON	Cnidaria I—Introduction to the Cnidaria and Ctenophora
ROBERT K. JOSEPHSON	Cnidaria II—Feeding, growth, function of the nematocysts
ROBERT K. JOSEPHSON	Cnidaria III—Nervous system and behavior
FRANK M. FISHER, JR.	Turbellaria and Trematoda
FRANK M. FISHER, JR.	Cestoda and Rhynchocoela
FRANK M. FISHER, JR.	Physiological considerations of the host-parasite relationship
W. D. RUSSELL HUNTER	Mollusca I—General molluscan organization. Functioning of mantle cavity in Gastropoda
W. D. RUSSELL HUNTER	Mollusca II—Gastropoda (continued). Mantle cavity and feeding mechanisms in Bivalvia

W. D. RUSSELL HUNTER	Short seminar—Some problems of mechanics in molluscs
W. D. RUSSELL HUNTER	Mollusca III—Adaptations in bivalves. Aspects of general physiology of gastropods and bivalves
W. D. RUSSELL HUNTER	Seminar—A history of the segmented mollusc
W. D. RUSSELL HUNTER	Mollusca IV—Functional morphology in Amphineura, Cephalopoda and minor groups
W. D. RUSSELL HUNTER	Seminar—The evolution of and physiological variation in the molluscs of fresh waters
IRWIN W. SHERMAN	Annelida I—Introduction: history, embryology, taxonomy, general characteristics, reproduction
IRWIN W. SHERMAN	Annelida II—Settling patterns, regeneration, feeding mechanisms, hydrostatic skeleton
IRWIN W. SHERMAN	Annelida III—Respiration, osmoregulation, nervous system and behavior
ERIC L. MILLS	Arthropoda I—General features of arthropods. Introduction to crustacean structure
IRWIN W. SHERMAN	Invertebrate hemoglobins
ERIC L. MILLS	Arthropoda II—Crustacean structure, physiology and reproduction
ERIC L. MILLS	Arthropoda III—Crustacean functional morphology and evolution
ERIC L. MILLS	Arthropoda IV—Crustacean functional morphology and evolution
FRANK M. FISHER, JR.	Ectoprotea—Entoprocta
FRANK M. FISHER, JR.	Aschelminthes
NORMAN MILLOTT	Asteroidea
NORMAN MILLOTT	Development of echinoids. Ophiuroidea
NORMAN MILLOTT	Holothuroidea. Crinoidea
A. FARMANFARMAIAN	Protochordata I
ERIC L. MILLS	The biology of an amphipod crustacean sibling species pair
A. FARMANFARMAIAN	Protochordata II
JAMES W. LASH	Ascidian metamorphosis
GEORGE G. HOLZ, JR.	Porifera
GEORGE G. HOLZ, JR.	The nature of the Protozoa
GEORGE G. HOLZ, JR.	Mastigophora I
GEORGE G. HOLZ, JR.	Mastigophora II
NORMAN MILLOTT	Seminar—Light sensitivities in echinoderms
GEORGE G. HOLZ, JR.	Sarcodina I
GEORGE G. HOLZ, JR.	Sarcodina II
FREDERIK B. BANG	Invertebrate pathology
GEORGE G. HOLZ, JR.	Ciliophora
ROBERT R. HESSLER	Miscellaneous studies on an obscure crustacean from Nobska Beach, <i>Decrocheilocaris typicus</i>
W. D. RUSSELL HUNTER	One approach to the zooplankton

MARINE ECOLOGY

I. CONSULTANTS

MELBOURNE R. CARRIKER, Marine Biological Laboratory
 BOSTWICK H. KETCHUM, Woods Hole Oceanographic Institution
 EDWIN T. MOUL, Rutgers University
 JOHN H. RYTHER, Woods Hole Oceanographic Institution

II. INSTRUCTORS

- W. ROWLAND TAYLOR, Department of Oceanography and The Chesapeake Bay Institute,
The Johns Hopkins University, in charge of course
DENNIS J. CRISP, Marine Science Laboratory, University College of North Wales, U. K.
LAWRENCE B. SLOBODKIN, Department of Zoology, University of Michigan
RICHARD A. BOOLOOTIAN, Department of Zoology, University of California, Los Angeles
FRANK E. ROUND, Department of Botany, University of Bristol, England (Joint appointment with Department of Marine Botany)
HOWARD L. SANDERS, Woods Hole Oceanographic Institution

III. SPECIAL LECTURERS

- LUIGI PROVASOLI, Haskins Laboratories, New York
PETER H. KLOPPER, Department of Zoology, Duke University

IV. LABORATORY ASSISTANTS

- MARGARET C. LLOYD, University of Michigan
BARRY M. HEATFIELD, University of California, Los Angeles

V. LECTURES

- | | |
|-----------------------|--|
| W. ROWLAND TAYLOR | Introduction to environmental biology |
| LAWRENCE B. SLOBODKIN | Classical models of population growth and competition |
| LAWRENCE B. SLOBODKIN | Experimental analyses of population growth and competition |
| LAWRENCE B. SLOBODKIN | Intrinsic rates of natural increase and reproductive value |
| LAWRENCE B. SLOBODKIN | Predator-prey interaction and energy flow |
| LAWRENCE B. SLOBODKIN | Evolutionary strategy |
| LAWRENCE B. SLOBODKIN | Species abundance distributions I |
| LAWRENCE B. SLOBODKIN | Species abundance distributions II |
| LAWRENCE B. SLOBODKIN | Biostatistics 1. Elementary concepts of errors and variance |
| LAWRENCE B. SLOBODKIN | Biostatistics 2. Binomial and poisson distributions |
| LAWRENCE B. SLOBODKIN | Biostatistics 3. Binomial and poisson distributions |
| LAWRENCE B. SLOBODKIN | Biostatistics 4. Normal distribution and variance analysis |
| LAWRENCE B. SLOBODKIN | Biostatistics 5. Non-parametric tests |
| W. ROWLAND TAYLOR | Zonation on rocky shores |
| VICTOR A. ZULLO | Systematics and ecology |
| W. ROWLAND TAYLOR | Chemistry and physics of sea water |
| W. ROWLAND TAYLOR | Radiant energy in the marine environment |
| W. ROWLAND TAYLOR | Phytoplankton organisms I: Diatoms |
| W. ROWLAND TAYLOR | Phytoplankton organisms II: Diatoms |
| W. ROWLAND TAYLOR | Phytoplankton organisms III: Dinoflagellates |
| W. ROWLAND TAYLOR | Primary productivity I |
| W. ROWLAND TAYLOR | Primary productivity II |
| RICHARD A. BOOLOOTIAN | Aspects of coral atoll ecology |
| RICHARD A. BOOLOOTIAN | Types of food utilization by marine organisms with emphasis on feeding adaptations |
| RICHARD A. BOOLOOTIAN | <i>Arbacia punctulata</i> populations of Falmouth Harbor |
| RICHARD A. BOOLOOTIAN | Reproductive biology of marine organisms: general patterns |
| RICHARD A. BOOLOOTIAN | Reproductive cycles of marine organisms |
| RICHARD A. BOOLOOTIAN | Reproductive physiology of the echinoid, <i>Strongylocentrotus purpuratus</i> |

RICHARD A. BOOLOOTIAN	Relation of nutrition to reproduction in <i>S. purpuratus</i>
RICHARD A. BOOLOOTIAN	The circulatory system of <i>S. purpuratus</i> and its relation to nutrition and reproduction
V. G. TURNER	The effects of crowding on growth and development of <i>S. dröbachiensis</i>
L. PROVASOLI	Culturing of algae in synthetic media I
L. PROVASOLI	Culturing of algae in synthetic media II
FRANK E. ROUND	Marine phytoflagellates with scales
HOWARD L. SANDERS	Animal sediment relationship
DONALD RHODES	Biogenic reworking of intertidal and subtidal sediments of the Cape Cod region
HOWARD L. SANDERS	Structure of a soft bottom community and some remarks on organic molecules as a food source for benthic animals
HOWARD L. SANDERS	Salinity, hydrography and the distribution of estuarine animals
FRANK E. ROUND	Migration rhythms of intertidal benthic diatoms
HOWARD L. SANDERS	Time, latitude and the structure of marine benthic communities and remarks on the deep-sea benthos
DENNIS J. CRISP	The role of pelagic larvae
DENNIS J. CRISP	Free-swimming stages and the problem of transition
DENNIS J. CRISP	The cyprid, a model settler ; and others
DENNIS J. CRISP	Habitat selection I. Choice of deposits
DENNIS J. CRISP	Habitat selection II. Chemical inducements and a novel chemical sense
DENNIS J. CRISP	Territorial behavior
R. S. SCHELTEMA	Pelagic larvae of the North Atlantic
FRANK E. ROUND	The history of a marine basin
PETER H. KLOPPER	On the classification of behavior
PETER H. KLOPPER	Seminars in environmental biology on the causes of tropical species diversity
PETER H. KLOPPER	Imprinting: General introduction
PETER H. KLOPPER	Imprinting: Sexual selection in birds
PETER H. KLOPPER	Maternal imprinting in mammals
*DENNIS J. CRISP	Ecology of marine larval settling

* Post-course lecture.

SYSTEMATICS-ECOLOGY PROGRAM

THE STAFF

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Resident Ecologist: ROBERT H. PARKER

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Visiting Investigators in Residence: RICHARD A. BOOLOOTIAN, LOUISE BUSH, DUANE HOPE, E. T. MOUL, DONALD J. ZINN

Secretaries: KAY CRAM, HAZEL F. SANTOS

Artists: DIANE JOHNSON, RUTH VON ARX

Captain of Research Vessel: JAMES P. W. OSTERGARD, JR.

Research Assistants: ANDREW L. DRISCOLL, DOUGLAS EBY, ROBERT KAUFMAN, CAROL KOURTZ, BARRY MARTIN, J. STEWART NAGLE, PETER J. OLDHAM, FRANKLYN OTT, PETER E. SCHWAMB, STEWART SANTOS, DEHN SOLOMON, JUNE THOMAS, SUSAN TRACY, VILIA TURNER, DIRK VAN ZANDT, ANTHONY WILLIAMS, HILARY M. WILLIAMS

I. SEMINARS (winter not included)

PAUL GALTISOFF	Anomalies and malformations in the shells of <i>Crassostrea virginica</i>
EDMUND SMITH	Review of boring bivalves
ROBERT F. GIBBS	Progress report on the Cape Cod National Seashore
JOHN ZEIGLER	Geology of the Cape Cod Region
GEORGE HAMPSON	The resurrection of <i>Nucula truncula</i>
WOLFGANG WIESER	Ecological approaches to the benthic meiofauna
CHARLES YENTSCH	Chlorophyll-phaeophytin relationships in the marine planktonic environment
ROBERT CONOVER	Assimilation of organic matter by zooplankton and the question of superfluous feeding
HARRY F. RECHER	Feeding efficiency and herons
ROBERT F. SISSON	Color transparencies of squid biology
LAWRENCE B. SLOBODKIN	Population dynamics and the escape reaction in Hydra
WILLIAM H. AMOS	Selected biophotographs of Delaware benthic and littoral invertebrates
FRANK E. ROUND	History of marine basins
DENNIS J. CRISP	Effects of the cold winter of 1962-63 on British marine fauna
VILIA TURNER	The effects of diet and crowding on growth and development in <i>Strongylocentrotus droebachiensis</i> larvae
HORACE W. STUNKARD	The role of parasitism in animal ecology and systematics
ARTHUR H. CLARKE	Fresh-water mollusks of the American arctic water shed
ERIC L. MILLS	The ecology of deep-sea amphipod crustaceans of New England

THE LABORATORY STAFF

HOMER P. SMITH, General Manager

IRVINE L. BROADBENT, Office Manager
 MISS JANE FESSENDEN, Librarian
 ROBERT KAHLER, Superintendent, Buildings and Grounds

ROBERT B. MILLS, Manager, Department of Research Service
 CARL O. SCHWEIDENBACK, Manager, Supply Department

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MRS. JUDITH A. KECK
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MARINE BIOLOGICAL LABORATORY

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 FREDERICK E. THRASHER
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BAMMAN, BARBARA C., Princeton University
BARNETT, GERALD, The Johns Hopkins Medical School
BARNHILL, ROBERT, Miami University at Ohio
BARTELS, EVA, Columbia University
BAUER, JOHN J., University of Miami School of Medicine
BELL, ALLEN, Upstate Medical Center
BENES, MARY, Brandeis University
BENISEK, MARY V., University of Michigan
BENNETT, JUDITH ANN, Syracuse University
BERKOWITZ, DANIEL M., New York University Medical School
BERKOWITZ, ELLEN M., New York University Medical School
BIKLE, DANIEL, Harvard University
BILLIAR, JOANNE, Harvard Medical School
BOCK, MARION, Christie Hospital, Manchester, England
BODIAN, HELEN, Goucher College
BOOKMAN, CHARLES A., Barlow School and Columbia University, College of Physicians and Surgeons
BRADLEY, JOAN C., Drew University
BRADY, FRANCINE, Syracuse University
BURKE, DONALD SCOTT, Western Reserve University
BUSSE, JOHN H., University of Rhode Island
CAROL, JOAN, Columbia University
CAROLAN, ROBERT M., Dartmouth Medical School
CHAFFEE, RICHARD BATES, JR., Syracuse University
CLARK, JOHN I., Dartmouth College
CLAYBROOK, MAJORIE W., Columbia University
COLLINS, SANDRA ELAINE, Smith College
CONOVER, SHIRLEY, National Institutes of Health
CONRAD, GARY W., Yale University
CORFF, SONDR, Western Reserve University
CRAWFORD, CAROLYN, Connecticut College
DACEY, JOAN F., Harvard University
DAVIDSON, HAROLD, National Institutes of Health
DENGINER, SUSAN, College of St. Mary of the Springs
DOANE, MARSHALL G., University of Maryland
DONALDSON, DONALD JAY, Tulane University
DONLEY, CLARK, The Johns Hopkins University
DRESCHER, PATRICIA, Columbia University
DRISCOLL, ANDREW L., Systematics-Ecology Program, Marine Biological Laboratory
DYRO, FRANCES M., University of Maryland School of Medicine
EBY, DOUGLAS, Boston University
EGAR, MARGARET W., Western Reserve University
EISENBERG, HENRY, Columbia University
EISENBERG, JAMES P., Colby College
ELDER, HUGH Y., Glasgow University, Scotland
FASS, SIMCHA U., Massachusetts Institute of Technology
FERNANDEZ, HECTOR R., Yale University
FINDLEY, DAVIS, University of Tennessee
FISH, CHERIE LYN, Smith College
FITZJARRELL, AUSTIN T., Tulane University
FOLDES, PAUL, Lehigh University
FORAN, ELIZABETH H., Smith College
FRANKLIN, LUTHER EDWARD, University of Miami
FREEMAN, SALLIE BOINEAU, Emory University

FUSARI, MARGARET HELENE, Boston University
GARDNER, GARY, Oberlin College
GATEFF, ELISABETH, Western Reserve University
GIORDANO, VICKI LYNN, University of Delaware
GOLDIZEN, VERNON C., Western Reserve University
GOLDMAN, ROBERT D., Princeton University
GOLDSTONE, ELLEN, Upstate Medical Center
GOODMAN, ROSANNE, The Johns Hopkins University
GORMAN, JESSICA A., University of California, Los Angeles
GORMAN, JOHN, University of California, Los Angeles
GRANGER, RONALD EUGENE, The Johns Hopkins University
GREBE, STEPHEN CHARLES, Oakland University
GROSS, GARY, Columbia University, College of Physicians and Surgeons
HABAS, LINDA B., American Museum of Natural History
HACKETT, PAUL ROGER, Western Reserve University
HAINES, MICHAEL F., Syracuse University
HAROSI, FERENC, The Rockefeller University
HARRIS, EDWARD M., Duke University
HARTMANN, JOHN F., University of Toronto, Canada
HEATFIELD, BARRY M., University of California, Los Angeles
HESS, MARJORIE B., Dartmouth Medical School
HEYMANN, PETER W., Washington University
HORN, DIANE, Stanford University
HORNIG, JOANNA, Radcliffe College
HUMPHREYS, SUSIE, Harvard University
HUNTER, WILLIAM BRUCE, University of California, Santa Barbara
IMLAY, MARC JAMES, Northwestern University
ISENBERG, DAVID, Deerfield Academy
JACOBS, CAROL F., Brandeis University
JACOBSON, MARCUS, University of Edinburgh
JOB, DONALD D., University of Illinois
KAUFMAN, ROBERT G., Columbia University
KEHLENBECK, EDNA K., Syracuse University
KELLY, ROBERT, Mellon Institute
KEM, WILLIAM R., University of Illinois
KENNY, DIANNE, Simmons College and Boston University
KIEN, MARJA, Boston University
KIMBALL, FRANCES, Reed College
KIMMEL, CHARLES B., The Johns Hopkins University
KISSIL, GEORGE WM., University of Connecticut
KOHNE, DAVID, Purdue University
KORNB�ITH, GINA, Brown University
KRATOWICH, NANCY ROSALIE, Columbia University
KRAWCHENKO, JOHN, Syracuse University
LATTMAN, EATON E., The Johns Hopkins University
LEFKON, BRUCE WARREN, Columbia University
LEITNER, V. ENA, Smith College
LENNOX, EDWIN S., Salk Institute for Biological Studies
LENTZ, JUDITH P., Yale University
LENTZ, THOMAS, Yale University
LIEB, KATHARINE H., University of Illinois
LIPSKY, DAVID, Columbia University
LITNA, ELIZABETH M., Carnegie Institution of Washington
LLOYD, MARGARET C., University of Michigan
LOWE, LOUISE, University of Toronto, Canada
MACDONALD, VICTOR W., Massachusetts Institute of Technology
MACDUFF, MARIE, New York University Medical Center
MAHAR, CONSTANCE QUINN, Syracuse University

MALCOLM, DOUGLAS, University of Edinburgh and University of Pittsburgh
MALONY, PETER C., Brown University
MATSUMOTO, YORIMI, University of Illinois
McDANIEL, JAMES S., Rice University
McGURN, ELEANOR A., Western Reserve University
MEISMER, DONALD M., University of Cincinnati
MERRILL, CHARLOTTE, Massachusetts Institute of Technology
MILLER, SANDRA M., University of Maryland School of Medicine
MOORAD, PHILIP J., JR., Princeton University
MORITZ, GISELA, Columbia University, College of Physicians and Surgeons
MOSCONA, M. H., University of Chicago
MUNDAY, JOHN CLINGMAN, JR., University of Illinois
MUNDAY, JUDITH BERRIEN, University of Illinois
NADOL, JOSEPH B., JR., Harvard College
NAGLE, J. STEWART, Systematics-Ecology Program, Marine Biological Laboratory
NASH, MARK S., Montgomery Junior College
OLDHAM, PETER J., Systematics-Ecology Program, Marine Biological Laboratory
ORTIZ, JOSE R., University of Chicago
OSTERGARD, JAMES P., Systematics-Ecology Program, Marine Biological Laboratory
OTT, FRANKLYN, University of Massachusetts
PAK, SUNG KWON, Princeton University
PARKER, CAROL ANN, University of Massachusetts
PHILIPPUS, PAMELA L., Colorado College
PICKERING, CAROLA, Oxford University
PIKE, JOHN DOUGLAS, Tufts University
PITTS, W. REID, JR., Harvard University Medical School
POCOCK, MARY WEIR, University of Texas
POST, CHARLES T., JR., Yale University
POTTER, DIANE, Columbia University
PRINCE, JEFFREY S., University of Massachusetts
RAAB, JACOB, University of Chicago
RAVITZ, MELVYN JAY, University of Vermont
RHODES, RUSSELL G., University of Tennessee
ROBERTSON, DOUGLAS R., Upstate Medical Center
ROBERTSON, LOLA E., American Museum of Natural History
ROBINSON, PEGGY, The Johns Hopkins University
ROSE, FLORENCE C., Tulane University
ROSEN, CHARLES T., University of Toronto, Canada
ROSENBERG, MARK J., Amherst College
ROSENBERG, MARTIN, New York University, Bellevue Medical Center
RUBENSTEIN, JUDITH, Columbia University, College of Physicians and Surgeons
RUFFING, FAITH E., Western Reserve University
SALTZMAN, ORAH, Barnard College
SANDLIN, RONALD A., National Institutes of Health
SAUL, DAVID, The Johns Hopkins University Medical School
SCHINDLER, GERDA, New York Medical College
SCHMIDT, JOHN HOWARD, Marquette University
SCHWAMB, PETER B., Systematics-Ecology Program, Marine Biological Laboratory
SELDIN, EDWARD B., Harvard Graduate School of Arts and Sciences
SETTLES, HARRY E., Tulane University
SHRAGER, PETER G., University of California, Berkeley
SLOANE, ELEANOR MARY, Mellon Institute
SLOANE, MOLLA REBECCA, Wellesley College
SMILACK, JERRY, The Johns Hopkins University Medical School
SMITH, BARRY HAMILTON, Massachusetts Institute of Technology
SOLOMAN, DEHN E., Kalamazoo College
SORENSEN, ROBERT L., University of Minnesota Medical School
SPECHT, PHILIP C., Syracuse University

SPENCER, REBECCA L., Mount Holyoke College
 SPIEGELMAN, MARJORIE J., University of Chicago
 SPINDEL, ROBERT, Columbia University, College of Physicians and Surgeons
 SPIRITES, R. S., Reed College
 SQUIRE, RICHARD D., North Carolina State University
 STARK, VIRGINIA ANN, Syracuse University
 STEPHENS, RAYMOND E., Dartmouth Medical School
 STONE, INGRID, Columbia University, College of Physicians and Surgeons
 SUDDITH, ROBERT L., Indiana University
 SUSSMAN, JOEL L., Massachusetts Institute of Technology
 SZULMAN, AARON E., University of Pittsburgh School of Medicine
 TAMM, SIDNEY L., University of Chicago
 TANNENBAUM, ALICE SUSAN, University of Maryland School of Medicine
 TASAKI, LYDIA, National Institutes of Health
 TERMAN, STANLEY A., Massachusetts Institute of Technology
 THABES, TAMARA M., University of Chicago
 THERRIEN, EDWARD, Syracuse University
 THOMAS, JUNE M., University of California, Los Angeles
 TRAGER, CAROLYN, Princeton University
 TUCKER, C. MICHAEL, Dartmouth College
 TURNER, VILIA, University of California, Los Angeles
 TUTTLE, JOAN, University of Rochester
 VALCOVIC, LAWRENCE R., North Carolina State University
 VAN AMBURG, SHARON ANN, Rice University
 VAN PRAAG, DINA, New York University
 VAN ZANDT, DIRK, Systematics-Ecology Program, Marine Biological Laboratory
 WECHSLER, JAMES A., Yale University
 WEINER, BEVERLY, Harvard University
 WENGER, KARLYNN L., Tulane University
 WERMUTH, BRUCE, Yale University
 WHITE, ERIC S., Dartmouth Medical School
 WILLIAMS, ANTHONY, Systematics-Ecology Program, Marine Biological Laboratory
 WILLIAMS, HILARY M., Systematics-Ecology Program, Marine Biological Laboratory
 WONG, KAI KONG, Oberlin College
 YAMAMOTO, YOSHIHIRO, Chiba University, Japan, and Newcomb College
 YENTSCH, ANNE E., Systematics-Ecology Program, Marine Biological Laboratory
 YUYAMA, SHUHEI, Western Reserve University
 ZELMAN, DAVID ALLEN, University of California, Berkeley
 ZERKIN, MILTON, Columbia University
 ZOLLINGER, WILLIAM, University of Pittsburgh

Library Readers, 1965

ALLEN, M. JEAN, Chairman and Professor of Biology, Wilson College
 AMBERSON, WILLIAM R., Marine Biological Laboratory
 ATWOOD, KIMBALL CHASE, Professor of Microbiology, University of Illinois
 BALL, ERIC G., Edward S. Wood Professor of Biochemistry, Harvard Medical School
 BERNÉ, ROBERT M., Professor of Physiology, Western Reserve University
 BODANSKY, OSCAR, Chief, Division of Enzymology and Metabolism, Sloan-Kettering Institute
 for Cancer Research
 BRIDGMAN, ANNA JOSEPHINE, Chairman and Professor of Biology, Agnes Scott College
 BUTLER, ELMER G., Henry Fairfield Osborn Professor of Biology, Princeton University
 CARLSON, ELOF AXEL, Assistant Professor of Zoology, University of California, Los Angeles
 CARLSON, FRANCIS D., Professor of Biophysics, The Johns Hopkins University
 CHASE, AURIN M., Professor of Biology, Princeton University
 CLARK, ARNOLD M., Professor of Biological Sciences, University of Delaware
 COHEN, SEYMOUR S., Chairman and Professor of Therapeutic Research, University of Pennsylvania School of Medicine

- COLLIER, J. R., Professor of Biology, Rensselaer Polytechnic Institute
- DAVIS, BERNARD D., Head of Department of Bacteriology and Immunology, Harvard Medical School
- EISEN, HERMAN N., Chairman and Professor of Microbiology, Washington University School of Medicine
- FLESCH, PETER, Research Professor of Dermatology, University of Pennsylvania
- GABRIEL, MORDECAI L., Professor of Biology, Brooklyn College
- GINSBERG, HAROLD S., Chairman and Professor of Microbiology, University of Pennsylvania School of Medicine
- GORLIN, RICHARD, Assistant Professor of Medicine, Harvard Medical School
- GREEN, JAMES W., Professor of Physiology, Rutgers, The State University
- GUREWICH, VLADIMIR, Assistant Professor of Clinical Medicine, New York College of Medicine
- HANDLER, PHILIP, James B. Duke Professor and Chairman, Biochemistry, Duke University
- HARLEY, REV. JAMES L., Associate Professor of Biology, Georgetown University
- HAUBRICH, ROBERT R., Assistant Professor of Biology, Denison University
- HURWITZ, CHARLES, Chief, Basic Science Laboratory and Assistant Professor of Microbiology, VA Hospital, Albany
- HURWITZ, JERALD, Professor of Molecular Biology, Albert Einstein College of Medicine
- ISSELBACHER, KURT J., Chief, Gastrointestinal Unit and Associate Professor of Medicine, Harvard Medical School and Massachusetts General Hospital
- JACOBS, MERKEL H., *Emeritus* Professor of Physiology, University of Pennsylvania
- KEOSIAN, JOHN, Professor of Biology, Rutgers, The State University
- LEVINE, R. P., Professor of Biology, Harvard University
- LOCHHEAD, JOHN H., Professor of Zoology, University of Vermont
- LONDON, IRVING M., Chairman and Professor of Medicine, Albert Einstein College of Medicine
- MALKIEL, SAUL, Research Associate, Children's Cancer Research Foundation, Inc.
- MARKS, PAUL A., Associate Professor, Columbia University
- MATEYKO, G. M., Associate Professor of Biology, New York University
- MCDONALD, SISTER ELIZABETH SETON, Chairman and Professor of Biology, College of Mt. St. Joseph on the Ohio
- MEISLER, RICHARD, Instructor in Philosophy, Antioch College and Columbia University
- NASON, ALVIN, Associate Director of McCollum-Pratt Institute and Professor of Biology, The Johns Hopkins University
- NOVIKOFF, ALEX B., Research Professor, Albert Einstein College of Medicine
- NYSTROM, RICHARD A., Assistant Professor of Biological Sciences, University of Delaware
- PERLMANN, GERTRUDE E., Associate Professor, The Rockefeller University
- READ, CLARK P., Professor of Biology, Rice University
- ROTH, JAY S., Professor of Biochemistry, University of Connecticut
- RUSSELL, HENRY D., Private Research
- RUTMAN, ROBERT J., Associate Professor of Chemistry, University of Pennsylvania
- SCHLESINGER, R. WALTER, Chairman, Department of Microbiology and Assistant Dean, Rutgers Medical School
- SHAPIRO, HERBERT, Associate Member of Research Laboratory, Albert Einstein Medical Center, Philadelphia
- SPIEGEL, MELVIN, Associate Professor of Biology, Dartmouth College
- SPRAGUE, JAMES M., Professor of Anatomy, University of Pennsylvania
- STEINHARDT, JACINTO, Professor, Georgetown University
- STURTEVANT, A. H., Thomas Hunt Morgan Professor of Biology, *Emeritus*, California Institute of Technology
- SWANSON, CARL P., Professor of Biology, The Johns Hopkins University
- WAKSMAN, BYRON H., Chairman, Department of Microbiology, Yale University
- WHEELER, GEORGE E., Associate Professor of Biology, Brooklyn College
- WIGGANS, DONALD S., Professor of Biochemistry, University of Texas Southwestern Medical School
- WILSON, THOMAS HASTINGS, Associate Professor of Physiology, Harvard Medical School
- WINTERS, ROBERT W., Professor of Pediatrics, Career Scientist, Health Research Council, Columbia University, College of Physicians and Surgeons

YNTEMA, CHESTER L., Professor of Anatomy, State University of New York, at Syracuse
 ZACKS, SUMNER I., Neuropathologist, Pennsylvania Hospital
 ZORZOLI, ANITA, Associate Professor of Physiology, Vassar College

Students, 1965

All students listed completed the formal course program, June 14–July 24. Asterisk indicates students completing Post-Course Research Program, July 25–September 4.

ECOLOGY

ALLEN, JAMES R., New York University
 *BAILEY, CLAUDIA F., Oberlin College
 *BOYER, JOHN F., University of Chicago
 CALABRESE, ANTHONY, University of Connecticut
 CARRIER, REV. YVAN, Fordham University
 DUVEL, WILLIAM A., JR., Tufts University
 GAINES, ARTHUR G., University of Rhode Island
 GOLDSMITH, MARIAN R., University of Pennsylvania
 HELLER, SUSAN P., Connecticut College
 KATONA, STEVEN K., Harvard College
 *LANG, JUDITH C., Yale University
 *LEVANDOWSKY, MICHAEL, Columbia University
 MALONE, PHILIP G., Western Reserve University
 *MCKIBBINS, DALE L., University of Arizona
 *MILLER, CHARLES B., Scripps Institution of Oceanography
 *TEITELBAUM, MAE, City College of New York
 *TOLDERLUND, DOUGLAS S., Columbia University

EMBRYOLOGY

*ATKINSON, BURR G., JR., University of Connecticut
 *BOLENDER, ROBERT P., Columbia University
 *CONE, MARGARET V., Mount Holyoke College
 *CRONIN, PATRICIA E., Dartmouth Medical School
 *DUBRUL, ERNEST F., Washington University
 *EISEN, ARTHUR Z., Harvard Medical School
 FUCHSMAN, LUCY, Harvard University
 *GERSHMAN, HOWARD S., The Johns Hopkins University
 *HAUSCHKA, PETER V., The Johns Hopkins University
 HERBERT, THOMAS J., The Johns Hopkins University
 *HOOPER, ABIGAIL W., Yale University
 *LARRIVEE, DENIS H., University of California, Berkeley
 *MACKINTOSH, FREDERICK R., Massachusetts Institute of Technology
 MARSHALL, RICHARD E., National Heart Institute
 *PEDERSON, THORU J., Syracuse University
 *RIDDLE, BARBARA J., Brandeis University
 *ROMANOFF, PHYLLIS S., The Rockefeller University
 *SINDELAR, WILLIAM F., Western Reserve University
 *VINSON, WALTER C., JR., Stanford University
 *WHISNANT, BETTY LYNN, Duke University

MARINE BOTANY

ANDREWS, HOLLINGS T., University of Kansas
 BARTHOLOMEW, KAREN E., University of California, Los Angeles

- *CONOVER, CAROL L., Drew University
- *DABNEY, MICHAEL W., Seton Hill College
- GROSS, RUDOLPH E., University of Maryland
- HALL, BARBARA SUE, University of Oregon
- HARGRAVES, PAUL E., University of Rhode Island
- HILTON, RICHARD L., JR., University of Arizona
- HOSTETTER, HEBER P., III, University of Arizona
- *LEMBI, CAROLE A., University of Tennessee
- *LOCKWOOD, LINDA G., Columbia University
- LYNN, RAYMOND I., Indiana University
- MACKIERNAN, GAIL B., College of William and Mary
- *MADSEN, MARCIA J., University of California, Davis
- MANN, JAMES EDWARD, University of Texas
- NALEPA, THOMAS F., Indiana University
- *OTT, FRANKLYN, University of Texas
- SEARS, JAMES R., University of Massachusetts
- *WALDREP, MARGARET, University of South Alabama
- *WATSON, MELVIN W., University of Louisville

PHYSIOLOGY

- *ASCHER, MICHAEL S., Dartmouth College Medical School
- *BLUMENTHAL, ALAN B., California Institute of Technology
- BURGESS, RICHARD R., Harvard University
- *CAMPBELL, RICHARD D., The Rockefeller University
- *COHN, CAL, Cornell University Medical School
- *CRAPO, LAWRENCE M., Harvard University
- CUSHMAN, SAMUEL W., The Rockefeller University
- *DUANE, WARREN, University of Illinois
- *FELDMAN, JERRY F., Princeton University
- *GAMOW, R. IGOR, University of Colorado Medical School
- *HENDRICKSON, WAYNE A., The Johns Hopkins University
- JARVIS, DEREK, University of Wisconsin Medical School
- KASCHE, VOLKER, Brandeis University
- *LEITER, EDWARD, Emory University
- LESKES, ANDREA, The Rockefeller University
- *MAKINEN, MARVIN W., University of Pennsylvania
- *MALAWISTA, STEPHEN E., Yale University School of Medicine
- *MEISLER, MIRIAM H., Ohio State University
- *MOYER, RICHARD W., University of California, Los Angeles
- MURGOLA, EMANUEL J., Yale University
- *PERL, WILLIAM, New York University Medical Center
- *POLLARD, HARVEY BRUCE, University of Chicago Medical School
- *REINER, ALBEY M., Harvard University
- ROTH, ROBERT, Brandeis University
- ROWLAND, LEWIS P., Columbia University, College of Physicians & Surgeons
- RUBINOW, SOL I., Cornell University
- *WHITE, HAROLD BANCROFT, III, Pennsylvania State University
- *WIKSELL, ANDRE JEAN, Washington University
- *WILLIAMS, JUDITH A. O., University of Illinois
- *WOLFE, JASON S., University of California, Berkeley

INVERTEBRATE ZOOLOGY

- BAST, SISTER EILEEN MARIE, University of Oklahoma
- *BATCHELLER, RUTHANNE, University of Massachusetts
- *BECK, ROBERT MARTIN, Cornell University

- BLAUG, MAURICE, University of Minnesota
 BRADLEY, ROSE MAE RITA, Dunbarton College
 BRIGGS, ELEANOR LIVINGSTON, Columbia University
 *BURKY, ALBERT JOHN, Syracuse University
 CAMHI, JEFFREY, Harvard University
 CLARK, GEORGE RICHMOND, California Institute of Technology
 DAWE, CLYDE JOHNSON, National Institutes of Health
 *DELCOMYN, FRED, University of Oregon
 FOWLER, SUSAN, Vassar College
 FRANKS, EDWIN CLARK, Ohio State University
 GEORGE, STEPHEN ANTHONY, The Johns Hopkins University
 *GITTINGER, JOHN WILLIAM, JR., Oberlin College
 HAIN, MICHAEL LAWRENCE, University of California, Davis
 *HATFIELD, CAROLYN SUE, University of California, Berkeley
 KUPFERBERG, PAUL LEWIS, Drew College
 LARSON, PRISCILLA ARLENE, Yale University
 LEE, SUE YING, University of Illinois
 LIND, NANCY KAY, Harvard University
 McIVER, SUSAN BERTHA, Washington State University
 MURPHY, NEIL FRANCIS, Washington & Jefferson College
 OBERLANDER, HERBERT, Western Reserve University
 OCHS, KATHLEEN FRANK, Washington University, St. Louis
 PAOLETTI, ROBERT ANTHONY, The Johns Hopkins University
 PHILIPPUS, PAMELA LEA, Colorado College
 POCOCK, MARY AVIS WEIR, University of Texas
 RACEY, LOUISE ADELE, Catholic University of America
 *RADER, JEANNE ISABELLE, Syracuse University
 ROSENBERG, MARK J., Amherst College
 SAMUEL, GUDISAY, University of Pennsylvania
 SETZLER, EILEEN MARIE, College of St. Mary of the Springs
 SMITH, DANIEL PAUL, Massachusetts Institute of Technology
 *SPICER, JEAN FRANCES, Pennsylvania State University
 THOMASSON, PATRICIA ANNE, University of Minnesota
 *TOBIAS, PETER STEPHEN, Oberlin College
 *TREBATESKI, SISTER MARY GABRIEL, University of Notre Dame
 UZZO, ANTHONY, JR., C. W. Post College
 WEHMAN, HENRY JOSEPH, The Johns Hopkins University

4. FELLOWSHIPS AND SCHOLARSHIPS, 1965

Bio Club Scholarship:

MAE TEITELBAUM, Ecology Course

Edwin Linton Memorial Endowment of the Washington and Jefferson College:

NEIL MURPHY, Invertebrate Zoology Course

Turtlox Scholarship Fund:

MARCIA MADSEN, Botany Course

5. TRAINING PROGRAMS

FERTILIZATION AND GAMETE PHYSIOLOGY TRAINING PROGRAM

I. INSTRUCTORS

CHARLES B. METZ, University of Miami, in charge of program
 C. R. AUSTIN, Tulane University, Delta Regional Primate Center
 JOHN BIGGERS, University of Pennsylvania

LUTHER E. FRANKLIN, University of Miami
 ALBERTO MONROY, University of Palermo, Italy
 LEONARD NELSON, Emory University

II. CONSULTANT

HAROLD JACKSON, Christie Hospital and Holt Radium Institute, Manchester, England

III. TRAINEES

BARROS, CLAUDIO, Tulane University
 BORISY, GARY G., University of Chicago
 BROWN, GEORGE G., Virginia Polytechnic Institute
 CLAYBROOK, JAMES R., Oregon Regional Primate Research Center
 FAGAN, LINDA, Bryn Mawr College
 GREGG, KENNETH W., Emory University
 HAND, GEORGE S., JR., University of North Carolina
 HINSCH, GERTRUDE W., Mount Union College
 HOLMAN, JOSEPHINE A., University of New Hampshire
 LEHRER, HARRIS I., Brandeis University
 LONG, JOHN A., Harvard University
 RADO, THOMAS A., Stanford University
 SCHUETZ, ALLEN W., University of Minnesota
 SINGH, UDAI N., McGill-Montreal General Hospital
 TOBEN, HOWARD R., University of Miami
 WHITTINGHAM, DAVID G., University of Pennsylvania

IV. LECTURES

JOHN SHAVER	Some Recent Work on the Immunobiology of Fertilization
SHELDON J. SEGAL	The Role of RNA in the Action of Estrogen
HERBERT STERN	Biochemical Studies of Gametogenesis in Plants
ROBERT W. NOYES	The Endometrium and Fertility
EVERETT ANDERSON	Some Comparative Aspects of the Fine Structure of Oocytes during Differentiation
GLENN W. SALISBURY	Ageing Phenomena in the Gamete
GEORGE W. NACE	The Role of Heterosynthesized and Autosynthesized Antigens in Fertilization in the Frog
A. ORVILLE DAHL	Pollen
WILLIAM L. WILLIAMS	Capacitation and Decapacitation of Rabbit Sperm
VINCENT ALLFREY	Active and Inactive States of Chromatin
HAROLD JACKSON	Effects of Certain Chemicals on Mammalian Spermatogenesis
CHARLES THIBAUT	<i>In vitro</i> Fertilization: A Technique in Mammalian Reproduction Research
MICHAEL HARPER	Egg Transport within the Fallopian Tube

NEUROPHYSIOLOGY TRAINING PROGRAM

I. INSTRUCTORS

S. W. KUFFLER, Harvard Medical School, in charge of program
 E. J. FURSHPAN, Harvard Medical School
 D. D. POTTER, Harvard Medical School

II. ASSISTANT

R. B. BOSLER, Harvard Medical School

III. RESEARCH ASSOCIATE

J. G. NICHOLLS, Harvard Medical School

IV. TRAINEES

DENIS BAYLOR, Yale University

SHIN-LO CHUNG, Harvard University

MONROE W. COHEN, McGill University, Montreal

EDWIN S. LENNOX, Salk Institute for Biological Studies

CHARLES R. MICHAEL, Harvard University

MYRNA B. MILLER, Western Reserve University

STEPHEN A. SCHLESINGER, The Johns Hopkins University

COMPARATIVE PHYSIOLOGY RESEARCH TRAINING PROGRAM

I. INSTRUCTORS

C. LADD PROSSER, University of Illinois, in charge of program

LEWIS H. KLEINHOLZ, Reed College

BERNARD C. ABBOTT, University of Illinois

WILLIAM T. W. POTTS, University of Birmingham, England

GABOR KALEY, New York Medical College

AARON JANOFF, New York University School of Medicine

II. ASSISTANTS

DONALD JOB, University of Illinois

FRANCES KIMBALL, Reed College

III. RESEARCH ASSOCIATE

Y. MATSUMOTO, University of Illinois

IV. TRAINEES

AUGENFELD, JOHN M., University of Oklahoma

DUNSON, WILLIAM A., University of Michigan

EVANS, DAVID H., Stanford University

FORD, ARTHUR C., Rider College

HAMBY, ROBERT J., University of Chicago

LEVIN, STEPHEN M., New York University

LIEB, WILLIAM R., University of Illinois

STRATTEN, WILFORD P., Indiana University

DONSHIK, PETER, New York Medical College

SUTTERLIN, ARNOLD M., University of Massachusetts

TAMAR, HENRY, Indiana State University

V. LECTURES

CLARK P. READ

PETER KLOPFER

JOHN ANDERSON

DENNIS J. CRISP

E. BAYLOR

WILLIAM POTTS

FRANK A. BROWN, JR.

Physiological Generalizations for Parasitism

On the Causes of Tropical Species Diversity

Aspects of Organ Regeneration in Echinoderms

Ecology of Marine Larval Settling

Meteorology and the Distribution of Plankton Near the Surface

Adaptation to Sea Water and Fresh Water

Geophysical Forces and Biological Rhythms

6. TABULAR VIEW OF ATTENDANCE, 1961-1965

	1961	1962	1963	1964	1965
INVESTIGATORS—TOTAL	458	494	490	512	572
Independent	256	279	261	273	284
Library Readers	49	56	51	47	62
Research Assistants	151	159	178	192	227
STUDENTS—TOTAL	130	121	124	126	128
Invertebrate Zoology	40	38	40	40	41
Embryology	21	20	20	20	20
Physiology	28	28	28	30	30
Botany	19	20	20	19	20
Ecology	22	15	16	17	17
TRAINEES—TOTAL				30	34
Nerve-Muscle				7	7
Comparative Physiology				7	11
Fertilization & Gamete				16	16
TOTAL ATTENDANCE	586	615	614	668	734
Less persons represented in two categories	1	4	5	7	4
	585	611	609	661	730
INSTITUTIONS REPRESENTED—TOTAL	132	118	120	140	218
By Investigators	107	81	83	117	142
By Students	70	57	73	23	76
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	3	3	4	0	0
By Students	0	2	0	0	0
FOREIGN INSTITUTIONS REPRESENTED	28	31	21	32	27
By Investigators	21	17	15	28	25
By Students	7	14	6	4	2

7. INSTITUTIONS REPRESENTED, 1965

American Museum of Natural History	Columbia University
Amherst College	Columbia University, College of Physicians & Surgeons
Antioch College	Connecticut College
Barlow School	Cornell University
Barnard College	Cornell University Medical College
Boston University	Dartmouth College
Boston University School of Medicine	Dartmouth Medical School
Brandeis University	Deerfield Academy
Brookhaven National Laboratory	Delta Regional Primate Research Center
Brooklyn College	Denison University
Brown University	Drew University
Bryn Mawr College	Duke University
California Institute of Technology	Dunbarton College
Carnegie Institution of Washington	The Albert Einstein College of Medicine
Catholic University of America	The Albert Einstein Medical Center, Philadelphia
Children's Cancer Research Foundation, Inc.	Emory University
City College of New York	Fordham University
Colby College	Georgetown University
College of Mount St. Joseph on the Ohio	Goldwater Memorial Hospital
College of St. Mary of the Springs	Goucher College
College of William and Mary	
Colorado College	

- Harvard College
 Harvard Medical School
 Harvard University
 Haskins Laboratories
 Illinois Teachers College North
 Indiana State University
 Indiana University
 Institute for Muscle Research
 Iowa State University
 The Johns Hopkins School of Hygiene & Public Health
 The Johns Hopkins School of Medicine
 The Johns Hopkins University
 Kalamazoo College
 King Ranch Laboratories
 Lehigh University
 Marquette University
 Massachusetts General Hospital
 Massachusetts Institute of Technology
 Mellon Institute
 Miami University of Ohio
 Montgomery Jr. College
 Mount Holyoke College
 The Mount Sinai Hospital
 Mount Union College
 National Cancer Institute
 National Heart Institute
 National Institute of Arthritis & Metabolic Diseases
 National Institutes of Health
 National Science Foundation
 Neurological Institute
 Newcomb College
 New York College of Medicine
 New York University
 New York University-Bellevue Medical Center
 New York University College of Dentistry
 New York University Medical Center
 North Carolina State University
 Northwestern University
 Oakland University
 Oak Ridge National Laboratory
 Oberlin College
 Ohio State University
 Oregon Regional Primate Research Center
 Oxford University
 Pennsylvania Hospital
 Pennsylvania State College
 Pennsylvania State University
 C. W. Post College
 Princeton University
 Providence College
 Purdue University
 Putnam Memorial Hospital, Institute for Medical Research
 Queens College of the City University of New York
 Radcliffe College
 Radcliffe Graduate Center
 Reed College
 Rensselaer Polytechnic Institute
 Rice University
 Rider College
 The Rockefeller University
 Russell Sage College
 Rutgers Medical School
 Rutgers, The State University of New Jersey
 Salk Institute for Biological Studies
 Agnes Scott College
 Scripps Institution of Oceanography
 Seton Hill College
 Simmons College
 Single Cell Research Foundation, Inc.
 Sloan-Kettering Institute for Cancer Research
 Smith College
 South Oaks Research Foundation, Inc.
 Stanford University
 State University of New York at Syracuse
 Syracuse University
 Syracuse University Upstate Medical Center
 Temple University
 The City University of New York, Brooklyn College
 The Institute for Cancer Research, Philadelphia
 Tufts University
 Tulane University
 Tulane University School of Medicine
 U. S. Bureau of Commercial Fisheries
 University of Arizona
 University of California, Berkeley
 University of California, Davis
 University of California, Los Angeles
 University of California, Riverside
 University of California, San Francisco
 University of Chicago
 University of Chicago Medical School
 University of Cincinnati
 University of Colorado Medical School
 University of Connecticut
 University of Connecticut School of Dental Medicine
 University of Delaware
 University of Illinois
 University of Illinois School of Medicine
 University of Iowa
 University of Kansas
 University of Louisville
 University of Maryland
 University of Maryland Medical School
 University of Massachusetts
 University of Miami
 University of Miami Institute of Molecular Evolution

University of Miami School of Medicine	University of Tennessee
University of Michigan	University of Texas
University of Minnesota	University of Texas, Southwestern Medical School
University of Minnesota Medical School	University of Vermont
University of New Hampshire	University of Virginia
University of North Carolina	University of Wisconsin
University of North Carolina School of Medicine	Vassar College
University of Notre Dame	Veterans Administration Hospital, Albany
University of Oklahoma	Veterans Administration Hospital, Brooklyn
University of Oregon	Washington & Jefferson College
University of Pennsylvania	Washington State University
University of Pennsylvania, Johnson Research Foundation	Washington University
University of Pennsylvania School of Medicine	Washington University School of Medicine
University of Pittsburgh	Wellesley College
University of Pittsburgh School of Medicine	Western Reserve University
University of Rhode Island	Western Reserve University School of Medicine
University of Rochester School of Medicine & Dentistry	Wilson College
University of South Alabama	Woods Hole Oceanographic Institution
University of South Florida	Yale University
	Yale University School of Medicine

FOREIGN INSTITUTIONS REPRESENTED, 1965

Bedford College, University of London	University of Birmingham, England
Chiba University, Japan	University of Brussels, Belgium
Christie Hospital, Manchester, England	University of Chile
Glasgow University, Scotland	University College, London
Kumamoto University, Japan	University of Edinburgh, Scotland
Leicester University, England	University of Gdaush, Poland
Marine Science Laboratory, University College of North Wales	University of Helsinki, Finland
McGill-Montreal General Hospital, Canada	University of Ljubljana, Poland
McGill University, Canada	University of Oslo, Norway
Pahlavi University, Iran	University of Saarbrucken, Germany
Queen's University, Canada	University of Sydney, Australia
St. George's Hospital Medical School, London	University of Toronto, Canada
Tokyo Medical & Dental University	University of Waterloo, Canada

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Atomic Energy Commission	Olin Matheson Charitable Trust
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Dr. Ethel Browne Harvey	Wallace Laboratories
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8. FRIDAY EVENING LECTURES, 1965

July 2

HOWARD K. SCHACHMAN The Subunit Structure of Proteins in Terms of
University of California, Berkeley Their Functions

July 8, Thursday

LESLIE F. NIMS Membranes, Metabolism and Material Transfer
Alexander Forbes Lecturer at the MBL
Brookhaven National Laboratory

July 9

LESLIE F. NIMS Galvani, Volta and Bioelectricity

July 16

ALEXANDER HOLLAENDER Studies in Radiation Biology at the Oak Ridge
Oak Ridge National Laboratory National Laboratory

July 23

HOWARD A. SCHNEIDERMAN The Hormonal Control of Insect Development
Western Reserve University

July 30

NORMAN MILLOTT The Enigmatic Echinoids
Bedford College, University of London

August 6

JOHN W. KANWISHER Thermal and Respiratory Physiology of
Woods Hole Oceanographic Whales and Porpoises
Institution

August 13

LAURI SAXÉN The Mechanism of Kidney Tubulogenesis
University of Helsinki
Senior Lalor Fellow at the MBL

August 20

SHINYA INOUÉ Exploration of the Living Cell with Polarized
Light

August 27

PHILIP B. ARMSTRONG Behavior in Developing Embryos
State University of New York
College of Medicine at Syracuse

9. TUESDAY EVENING SEMINARS, 1965

July 13

GEORGE SZABÓ Suntanning

M. PATHAK

W. C. QUEVEDO, JR.

W. S. VINCENT Ribosome Synthesis in Synchronous Yeast
Cultures

ROBERT G. FAUST Action of Bile Salts on ATP-ase Activity of
Mucosal Homogenates from Rat Jejunum and
Ileum

July 20

- ARNE LØVLIE Photosynthesis during the Cell Cycle of *Englena gracilis*
 M. FINGERMAN Neuroendocrine Control of the Crustacean
 Y. YAMAMOTO Hepatopancreas
 D. E. PHILPOTT Intracellular Aggregates and Granules of *Limulus* Gill Cartilage
 P. PERSON

July 27

- JOHN D. PALMER A Biological Rhythm in *Englena*
 BERND LINDEMANN Time-Independent Negative Sodium Conductance in the Surface Structure of Frog Skin Epithelium
 RICHARD L. MILLER Chemotaxis (?) of Coelenterate Sperm

August 3

- BERND LINDEMANN Effects of Direct Current Passing Through a Model Epithelial Cell
 RUTH JOHNSON Chemically Induced Orientation of the Growth of Malignant Cells *in vitro*
 SIDNEY LERMAN Properties of a Cryoprotein in the Ocular Lens
 LEWIS G. TILNEY The Role of Microtubules in the Formation and Maintenance of the Axopodia of *Actinosphaerium nucleofilum*
 DOUGLAS MARSLAND

August 10

- REUBEN TORCH The Effects of Actinomycin on RNA Synthesis in the Brackish-Water Ciliate, *Tracheloraphis* sp.
 W. A. HAGINS Photon Statistics and Central Mechanisms of
 F. HANSON Bioluminescence
 J. B. BUCK
 ROGER ECKERT Microsources of Luminescence in *Noctiluca*
 GEORGE REYNOLDS
 RICHARD CHAFFEE
 J. W. HASTINGS Light-induced Bioluminescence
 Q. H. GIBSON

August 17

- JAMES W. LASH Studies on Tunicate Metamorphosis
 JOHN R. REIGART, II
 DOUGLAS MARSLAND Synergism Between Colchicine and High Pressure in Regard to Anti-Mitotic Effects in Dividing Eggs (*Lytechinus variegatus*)
 R. E. KANE Identification and Isolation of the Mitotic Apparatus Protein
 R. E. STEPHENS Characterization of the MA Protein and its Subunits

10. MEMBERS OF THE CORPORATION, 1965

Including Action of 1965 Annual Meeting

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V. REPORT OF THE LIBRARIAN

This year the old office section of the Library was changed to a small reading reference room. Built-in bookcases line the walls and special racks hold the week's receipts of journals. Leather furniture, carpet and draperies make it a comfortable and attractive room. New chairs were ordered for the large main reading room and plans were made for 8 carrels and a typing room on the third floor.

The 1965 "Serial Publications" list was published. In 300 pages this book lists the nearly 3,600 separate journal titles held by the Library. A large number of libraries have purchased this publication and it has almost doubled our inter-library loan requests. During the year we received and serviced 1,580 requests from colleges and universities throughout the country. We also received many requests from Germany, India and Japan. We made 166 requests from other libraries for the use of investigators here.

One thousand and eight hundred volumes were sent to the bindery in 1965 and we added 112 new journals to our collection. We made an exact count of the number of volumes in the library and our total holding is now 126,423. This figure does not include our 235,000 reprints.

Total number of serial titles in library	3,675
Number received currently	2,025
On subscription	840
On exchange	899
On gift basis	286
Total number of reference books and monographs	15,795
Number added in 1965	411
Received from book exhibits	109
Total number of reprints	235,663
Number added in 1965	4,312

Respectfully submitted,
 JANE FESSENDEN,
Librarian

VI. REPORT OF THE TREASURER

The market value of the general Endowment Fund and the Library Fund at December 31, 1965, amounted to \$2,502,903 as against book value of \$1,261,842. This compares with values of \$2,370,890 and \$1,235,860, respectively, at the end of the preceding year. The average yield on the Securities was 3.34% of the market value and 6.62% of the book value. Uninvested principal cash in the above accounts at the end of the year was \$1,878. Classification of the securities held in the Endowment Fund appears in the Auditor's report.

The market value of the pooled securities as of December 31, 1965, was \$702,137 as compared with \$580,677 being the market value as of December 31, 1964, the increase being the result of addition of funds from Herbert W. Rand Fellowship, Mellon Foundation and Mary Rogick Fund. Uninvested principal cash at the end of the year was \$13,415. Book value of securities in this account at the beginning of this year was \$562,547 compared with \$646,802 at the close of 1965. The average yield on market value was 2.93% and 3.18% on book value.

The proportionate interest in the Pool Fund Account of the various Funds as of December 31, 1965, is as follows:

Pension Funds	21.217%
General Laboratory Investment	28.909%
F. R. Lillie Memorial Fund	3.151%
Anonymous Gift	1.080%
Other:	
Bio Club Scholarship Fund	.815%
Rev. Arsenius Boyer Scholarship Fund	.998%
Gary N. Calkins Fund	.937%
Allen R. Memhard Fund	.182%
Lucretia Crocker Fund	3.411%
E. G. Conklin Fund	.576%
Jewett Memorial Fund	.303%
M. H. Jacobs Scholarship Fund	.411%
Herbert W. Rand Fellowship	22.167%
Mellon Foundation	13.760%
Mary Rogick Fund	2.083%

Donations from the MBL Associates for 1965 were \$7,385.00, as compared with \$4,830.00 for 1964. Unrestricted gifts from foundations, societies and companies amounted to \$53,950.

During the year, we administered the following grants:

<i>Investigators</i>	<i>Training</i>	<i>MBL Institutional</i>
12 NIH	3 NIH	3 NIH
4 NSF	2 NSF	2 NSF
1 Ford		2 ONR
2 ONR		1 AEC
1 Commonwealth		1 Ford
1 Whitehall		
21	5	9

The rate of overhead on grants to investigators is 20% based on the amount expended. The overhead on these grants for this year amounted to \$94,791 as compared with \$81,239 for the preceding year. A new formula for determining an indirect Cost Rate was introduced this year by NIH which computed our rate of overhead to be 34.3% of direct cost and 73.36% of salaries and wages.

The Lillie Fellowship Fund with a market value of \$158,524 and a book value of \$92,887, as well as the investment in General Biological Supply House with a book value of \$12,700, is carried in the Balance Sheet item "Other Investments."

The General Biological Supply House fiscal year ended June 30, 1965, and had a profit after taxes of \$275,080 as compared to \$309,651 in 1964, \$241,616 in 1963, \$302,657 in 1962, and \$302,851 in 1961.

During the period covered by this report the Marine Biological Laboratory received dividends from the General Biological Supply House of \$63,500 as against \$63,500 in 1964, \$42,164 in 1963, \$38,000 in 1962 and \$33,020 in 1961.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1965, the related statement of operating expenditures, income and current fund and statement of funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We examined and have reported on financial statements of the Laboratory for the year ended December 31, 1965.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1965, and 1964, and the results of its operation for the years then ended on a consistent basis.

The supplementary schedules included in this report were obtained from the Laboratory's records in the course of our examination and in our opinion, are fairly stated in all material respects in relation to the financial statements, taken as a whole.

Boston, Massachusetts
May 2, 1966

LYBRAND, ROSS BROS. AND MONTGOMERY

ALEXANDER T. DAIGNAULT,
Treasurer

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1965 and 1964

<i>Investments</i>		1965	1964
Investments held by Trustee:			
Securities, at cost (approximate market quotation 1965—\$2,502,903)		\$1,261,842	\$1,235,860
Cash		1,878	5,595
		<u>1,263,720</u>	<u>1,241,455</u>
Investments of other endowment and unrestricted funds:			
Pooled investments, at cost (approximate market quotation 1965—\$640,065) less \$5,728 temporary investment of current fund cash		522,591	479,344
Other investments		119,352	118,888
Cash		55,608	39,522
Accounts receivable		7,384	10,664
		<u>\$1,968,655</u>	<u>\$1,889,873</u>
<i>Plant Assets</i>			
Land, buildings, library and equipment (note)		5,481,019	5,136,289
Less allowance for depreciation (note)		1,449,145	1,378,887
		<u>4,031,874</u>	<u>3,757,402</u>
Construction in progress		41,253	109,215
Cash		90,015	
Short-term investments, at cost		50,000	192,360
		<u>\$4,213,142</u>	<u>\$4,058,977</u>
<i>Current Assets</i>			
Cash		35,596	79,637
Temporary investment in pooled securities		5,728	5,728
U. S. Treasury bills, at cost		74,210	96,045
Accounts receivable (U. S. Government, 1965—\$78,108; 1964—\$84,793) .		139,079	138,489
Inventories of supplies and Bulletins		35,493	33,401
Prepaid insurance and other		1,893	6,844
		<u>\$ 291,999</u>	<u>\$ 360,144</u>

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1965 and 1964

<i>Invested Funds</i>		
	1965	1964
Endowment funds given in trust for benefit of the Marine Biological Laboratory	\$1,263,720	\$1,241,455
Endowment funds for awards and scholarships:		
Principal	322,135	295,710
Unexpended income	24,400	14,289
	<u>346,535</u>	<u>309,999</u>
Unrestricted funds functioning as endowment	206,378	206,378
Retirement fund	135,952	123,298
Pooled investments—accumulated gain	16,070	8,743
	<u>\$1,968,655</u>	<u>\$1,889,873</u>
<i>Plant Funds</i>		
Funds expended for plant, less retirements	5,522,272	5,245,504
Less allowance for depreciation charged thereto	1,449,145	1,378,887
	<u>4,073,127</u>	<u>3,866,617</u>
Unexpended plant funds	140,015	192,360
	<u>\$4,213,142</u>	<u>\$4,058,977</u>
<i>Current Liabilities and Funds</i>		
Accounts payable and accrued expenses	53,563	33,315
Advance subscriptions	16,074	10,926
Unexpended grants—research	64,094	99,000
Unexpended balances of gifts for designated purposes	18,772	17,995
Current fund	139,496	198,908
	<u>\$ 291,999</u>	<u>\$ 360,144</u>

Note—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

STATEMENTS OF OPERATING EXPENDITURES, INCOME AND CURRENT FUND

Years Ended December 31, 1965 and 1964

<i>Operating Expenditures</i>		1965	1964
Research and accessory services	\$	294,798	\$ 293,396
Instruction		196,509	160,820
Library and publications (including book purchases— 1965, \$39,356; 1964, \$24,304)		102,595	80,416
Direct costs on research grants		514,709	493,890
Direct costs on institution support grants		161,669	150,450
		<u>1,270,280</u>	<u>1,178,972</u>
Administration and general		125,298	112,453
Plant operation and maintenance		131,885	139,684
Dormitories and dining		193,298	175,696
Additions to plant from current fund		128,448	5,045
		<u>1,849,209</u>	<u>1,611,850</u>
Less depreciation included in plant operation and dormitories and dining above but charged to plant funds		71,461	67,437
		<u>1,777,748</u>	<u>1,544,413</u>
<i>Income</i>			
Research fees		96,745	88,240
Accessory services (including sales of biological specimens— 1965, \$37,586; 1964, \$42,051)		107,204	114,181
Instruction fees		28,525	28,470
Library fees, Bulletins, subscriptions and other		53,687	49,393
Dormitories and dining income		149,754	133,759
Grants for support of institutional activities:			
Instruction and training		198,404	138,540
Support services		161,669	150,450
General		106,445	109,219
Reimbursements and allowances for direct and indirect costs on specific research grants		592,225	575,129
Gifts used for current expenses		66,368	22,135
Investment income used for current expenses		157,310	156,676
		<u>1,718,336</u>	<u>1,566,192</u>
Excess current income (expenditures)		(59,412)	21,779
Current fund balance January 1		198,908	177,129
Current fund balance December 31	\$	<u>139,496</u>	<u>\$ 198,908</u>

MARINE BIOLOGICAL LABORATORY

STATEMENT OF FUNDS

Year Ended December 31, 1965

	<i>Balance December 31, 1964</i>	<i>Gifts and Other Receipts</i>	<i>Invest- ment Income</i>	<i>Used for Current Expenses</i>	<i>Other Expendi- tures</i>	<i>Balance December 31, 1965</i>
Invested funds	\$1,889,873	\$ 78,513	\$169,627	\$ 154,291	\$ 15,067	\$1,968,655
Unexpended plant funds	\$ 192,360	50,000	7,944		110,289	\$ 140,015
Unexpended research grants	\$ 99,000	1,023,837		1,058,743		\$ 64,094
Unexpended gifts for designated purposes	\$ 17,995	72,052		66,368	4,907	\$ 18,772
Current fund	\$ 198,908	(59,412) (1)				\$ 139,496
		<u>\$1,164,990</u>	<u>\$177,571</u>	<u>\$1,279,402</u>	<u>\$130,263</u>	
Gifts		148,477				
Grants for research, training and support		1,023,837				
Appropriated from current income and other		22,496				
Net gain on sale of securities		29,592				
(1) Excess of current expenditures over income		(59,412)				
		<u>\$1,164,990</u>				
Expended for construc- tion and renovation of facilities					110,289	
Scholarship awards ...					1,101	
Payments to pensioners					14,578	
Other					4,295	
					<u>\$130,263</u>	

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1965

	<i>Cost</i>	<i>% of Total</i>	<i>Market Quotations</i>	<i>% of Total</i>	<i>Investment Income 1965</i>
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	\$ 4,993	.5	\$ 4,973	.2	\$ 5,008
Corporate bonds	657,502	62.5	631,091	30.7	21,432
Preferred stocks	54,422	5.2	57,000	2.8	2,300
Common stocks	334,791	31.8	1,363,906	66.3	41,384
	<u>1,051,708</u>	<u>100.0</u>	<u>2,056,970</u>	<u>100.0</u>	<u>70,124</u>
General Educational Board endowment fund:					
U. S. Government securities	18,953	9.0	18,405	4.1	1,033
Other bonds	115,718	55.1	111,604	25.0	4,515
Preferred stocks	15,641	7.4	14,125	3.2	805
Common stocks	59,822	28.5	301,799	67.7	7,088
	<u>210,134</u>	<u>100.0</u>	<u>445,933</u>	<u>100.0</u>	<u>13,441</u>
Total securities held by Trustee	<u>\$1,261,842</u>		<u>\$2,502,903</u>		<u>\$ 83,565</u>
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	32,984	6.3	31,782	5.0	1,681
Corporate bonds	160,391	30.4	153,798	24.0	5,086
Common stocks	334,944	63.3	454,485	71.0	13,859
	<u>528,319</u>	<u>100.0</u>	<u>\$ 640,065</u>	<u>100.0</u>	<u>20,626</u>
Less temporary investment of current fund cash	(5,728)				(246)
	<u>522,591</u>				<u>20,380</u>
Other investments:					
U. S. Government securities	27,943				1,128
Other bonds	15,029				749
Preferred stocks	3,728				130
Common stocks	58,887				65,768
Real estate	13,765				
	<u>119,352</u>				<u>67,775</u>
Total investments of other endowment and unrestricted funds	<u>\$ 641,943</u>				<u>88,155</u>
Total investment income					171,720
Custodian's fees charged thereto					(2,093)
Investments income distributed to funds ..					<u>169,627</u>
Plant investments:					
Federal agency and corporate bonds ..	<u>\$ 50,000</u>				<u>7,944</u>
Current investments:					
U. S. Treasury bills, due February 3, 1966	<u>\$ 74,210</u>				<u>2,773</u>
Temporary investment in pooled securities	<u>\$ 5,728</u>				<u>246</u>
					<u>3,019</u>
					<u>\$180,590</u>

CORRELATION OF LYSOSOMAL ACTIVITY AND INGESTION BY THE MANTLE EPITHELIUM^{1, 2}

GERRIT BEVELANDER AND HIROSHI NAKAHARA

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and The Bermuda Biological Station for Research*

Despite the fact that the structure and function of the molluscan mantle has occupied the attention of investigators for well over one hundred years the role of the outer mantle epithelium in the elaboration of the shell is as yet poorly understood. On the basis of histochemical and radioautographic studies (Bevelander and Benzer, 1948; Bevelander, 1952), it was shown that both mucus secreted by the mantle and Ca^{45} derived from the sea water environment were incorporated in the shell. Several other authors (Ojima, 1952; Wilbur, 1960; Kado, 1960; Tsujii, 1960) have also remarked on the fact that mucus and other formed substances play a significant role in shell formation. It was further shown by Nakahara (1962a) that Ca^{45} injected into the adductor muscle was incorporated into the mantle mucus which later became an integral part of the calcified nacre.

The fine structure of the mantle of *Fabulina* was described by Kawaguti and Ikemoto (1962). These authors contend that the outer surface of the mantle is made up of three cell types. Mention is made of the presence of microvilli, mitochondria, Golgi bodies, endoplasmic reticulum, but the presence of lysosomes is not mentioned. Furthermore no specific function of the various cell types described was suggested.

The above references, although by no means complete as to the number of published studies dealing with the structural and functional aspects of the mantle epithelium, do, however, indicate that the chief interest and concern of these investigations was the identification of various substances elaborated and secreted by the epithelium and their subsequent identification in the formed shell.

It was previously shown by Nakahara (1962) that the outer surface of the mantle of *Pinctada martensii* ingests carmine particles placed in the extrapallial fluid. In an attempt to clarify and amplify the above observations we have examined this problem in more detail, utilizing electron microscopy, histochemistry as well as experimental physiological procedures.

Briefly stated, this report deals with a description of the results obtained following the procedures mentioned above, by means of which we have demonstrated differential absorption by the outer surface epithelium of the mantle. A description of the intracellular localization of acid phosphatase, lipids and mucopolysaccharides at the light level and the detailed structure of the ingesting cell follows. Finally the locus of ingested material, together with other remarks concerning lysosomal activity in the mantle cells, is described.

¹ This investigation was supported (in part) by grant DE-01825-04, N.I.D.R., U.S.P.H.S.

² Contribution No. 385 from the Bermuda Biological Station.

MATERIALS AND METHODS

This study was carried out on the calico clam, *Macrocallista maculata*. Specimens were collected in Bermuda waters and for purposes of histological and E.M. studies the mantle was removed and fixed in (1) a 1% solution of osmium tetroxide in a phosphate buffer at a pH of 7.4 for one hour at 25° C. (2) Other specimens were fixed in a 6% solution of glutaraldehyde buffered in phosphate at pH 7.4 for 4-6 hours at 25° C. Following this treatment the tissues were repeatedly washed in phosphate buffer and fixed in osmium tetroxide for one hour as described above. Following fixation, tissues were routinely dehydrated, embedded in Araldite and were then sectioned for E.M. observations (thin sections) and also 1-2 μ sections which were stained with a 40% alcoholic solution of 1:1000 toluidine blue for observations with the light microscope.

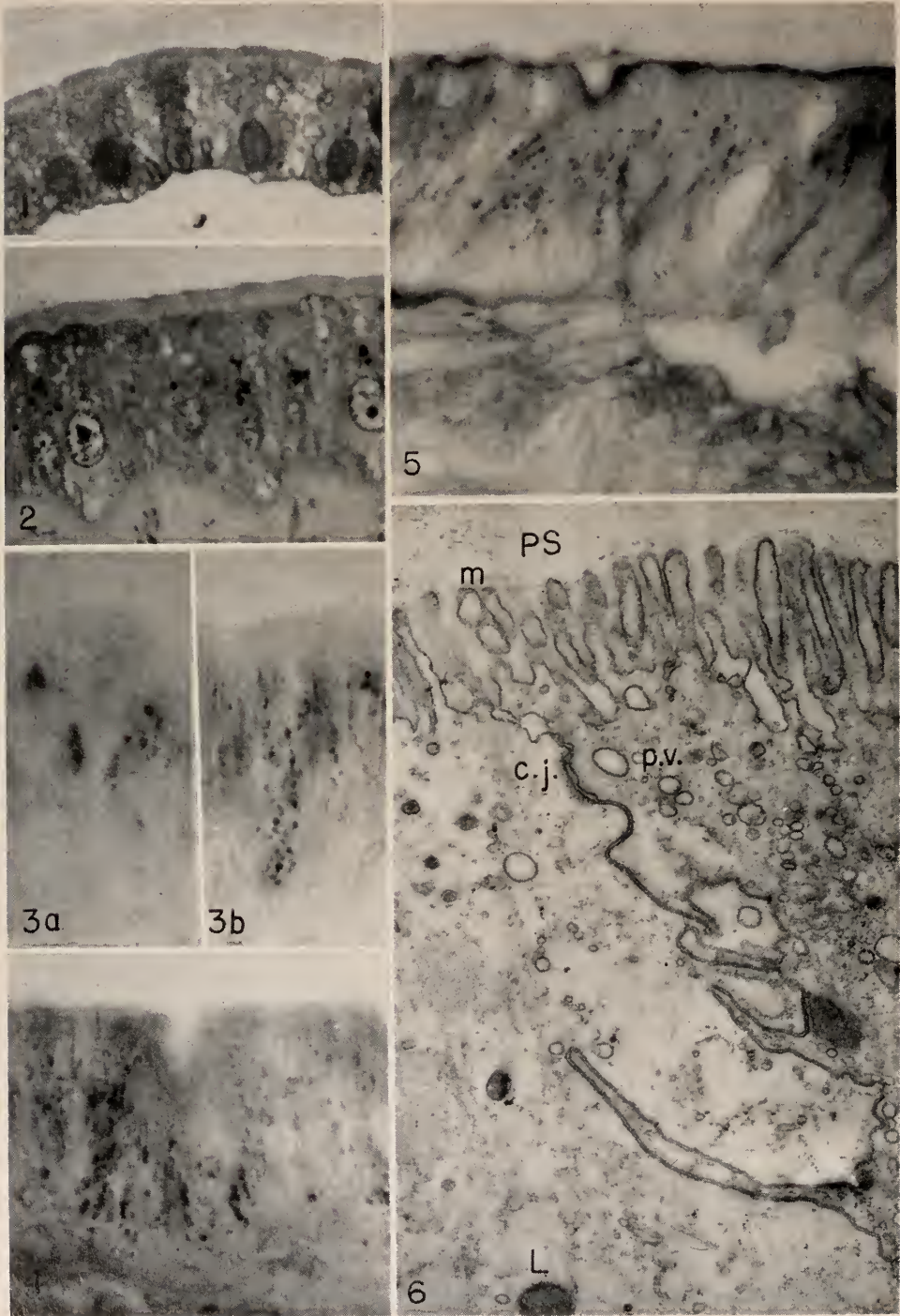
For the purposes of ascertaining the ability of the shell-forming epithelium to absorb particulate material the following procedures were carried out: 0.1 cc. of a 2% filtered sea water suspension of finely ground carmine particles was injected into the pallial space. Specimens were then placed in tanks furnished with running sea water at a temperature of 25-26° C. for a period of 1-3 days. At the termination of these periods they were removed from the tanks, the mantle was excised, fixed in Ca-formol, dehydrated and then sectioned for subsequent observation. This procedure was also carried out with several other specimens in which a sea water-colloidal gold (Hartman-Leddon) mixture was injected in place of the carmine. The colloidal gold mixture was prepared just prior to injection by adding equal amounts of aliquots of colloidal gold and sea water concentrated to 50% of their original volumes.

To aid in the identification of the ingestion sites, three histochemical procedures were utilized. They consisted in testing the mantle epithelium for the presence of acid phosphatase, according to the method of Gomori (1950), in the treatment of the mantle to ascertain the presence of lipids by the Sudan black B method (Chiffelle and Putt, 1950), and the PAS method to demonstrate the presence of PAS-positive reaction sites.

OBSERVATIONS AND RESULTS

When viewed by means of light microscopy, the cells of the outer fold appear typically columnar, containing basally located nuclei, folded cell membranes, numerous vacuoles and a prominent cuticular border (Fig. 1).

Examination of sections of the mantle epithelium following injection of carmine into the pallial space revealed uptake of dye particles throughout the entire surface of the outer epithelium of *Macrocallista*. The most pronounced uptake occurs in the epithelium of the outer surface of the outer fold, less pronounced in the thick region of the mantle distal to the base of the outer fold, while the remainder of the mantle epithelium (thin portion) exhibits still less dye uptake than the other regions. The localization of the dye is observed as granules of varying sizes, in some instances so small as to be hardly recognized by light microscopy; in others the granules are readily recognizable at a magnification of 400 $\times$ (compare Figures 1 and 2). In addition to the outer (shell) epithelium, numerous amoebocytes also exhibit marked uptake of the carmine. It should be noted that the distribution of



FIGURES 1-6.

the carmine particles observed in these cells varies considerably. One of the factors upon which this distribution is dependent is the time allowed for ingestion to occur.

Sections of the mantle examined following treatment for the demonstration of acid phosphatase show a localization of this enzyme in the epithelial cells to be similar in size and distribution to the localization of the carmine granules described above (Fig. 3a, b).

Examination of mantle epithelium stained with Sudan black B reveals the presence of lipids to be rather widely distributed, appearing as dark granules of approximately the same size and position as those observed in the epithelium exhibiting ingestion of carmine. Sections treated according to the PAS method and subsequently digested with saliva exhibit PAS-positive granules in the cytoplasm which correspond in size and location to granules observed by the other two methods mentioned above. Briefly, the localization of carmine, acid phosphatase, lipid and PAS-positive granules appears to be very similar (Figs. 4, 5). The presence of acid phosphatase, lipids and PAS-positive material in granules occurring in the cytoplasm is, according to Novikoff (1960), a criterion for the identification of lysosomes at the level of light microscopy.

In the paragraphs which follow we shall describe the structure of the epithelial cell as observed by the electron microscope. Although structural variations occur, the example we have chosen to illustrate exhibits most of the details which are characteristic of these cells. At the free surface one observes rather short microvilli which extend for a considerable distance into the underlying cytoplasm.

A prominent feature of these cells is the septate cell junction and the very pronounced folding of the cell membrane. Mitochondria are numerous and usually occupy a position in the distal half of the cell (Fig. 7). In several cells in this part of the mantle, the mitochondria appear to be undergoing degenerative changes, as shown by the poorly defined cristae and outer membranes. Ribosomes, cisternae and glycogen are much less prominent than in other regions of the mantle. The Golgi apparatus, not illustrated in our microphotograph, is also characteristic, usually appearing rather widely dispersed throughout the cell. The cells rest upon a well defined basement membrane and from this membrane scattered integumental fibers arise and extend to the free surface.

The presence of numerous micropynocytotic vesicles arising from the canaliculi

Figures 1-5 are microphotographs of epithelium of the mid-portion of the outer mantle fold.

FIGURE 1. Typical columnar epithelium of outer mantle fold, showing cuticle (microvilli) on surface, folded cell membranes, numerous vacuoles and prominent basally located nuclei. Araldite section, stained with toluidine blue. $\times 1600$.

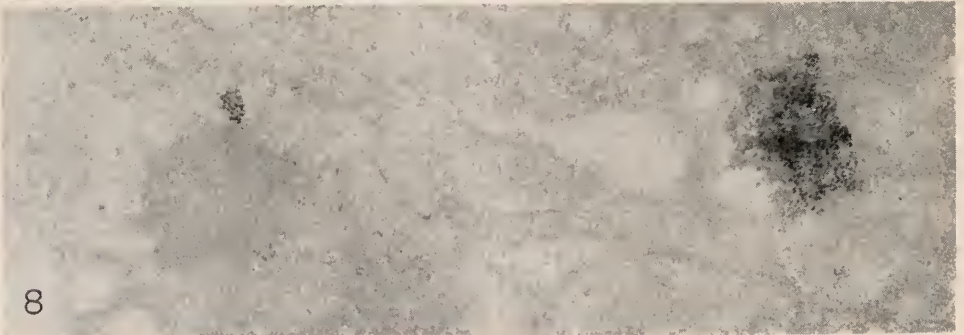
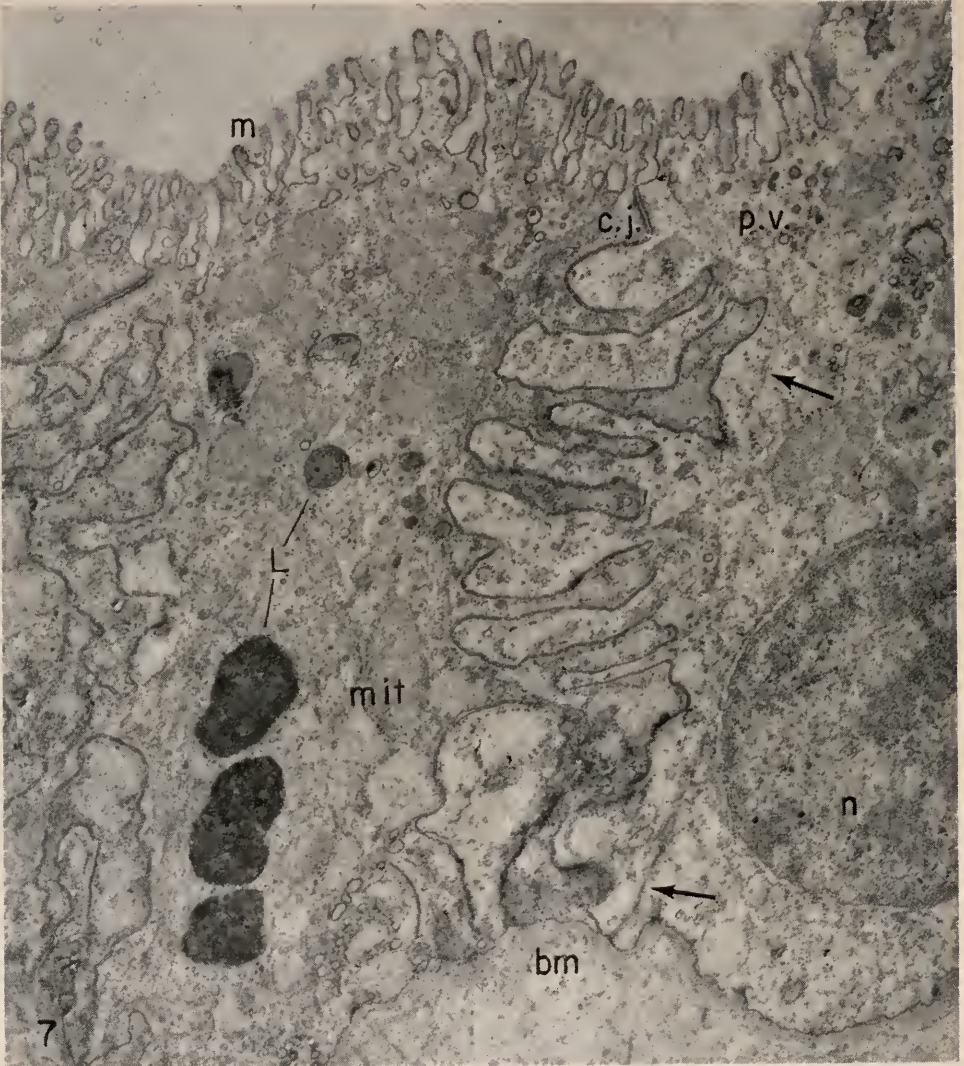
FIGURE 2. Section from specimen injected with carmine and fixed 24 hours later. Note distribution of fairly large dense granules indicating localization of carmine within the cell. Araldite section, unstained. $\times 1600$.

FIGURE 3, a and b. Adjacent sections from specimen treated to show the localization of acid phosphatase indicated by dark granules. These photos illustrate the variation in enzymatic activity in adjacent parts of the mantle. $\times 1600$.

FIGURE 4. Photograph of section fixed in Ca-formol and treated to show the localization of lipids indicated by the dark granules in the cytoplasm. $\times 1600$.

FIGURE 5. This section was digested with saliva and then treated to demonstrate PAS-positive material. Note distribution of cytoplasmic granules. $\times 1600$.

FIGURE 6. Electron micrograph of part of cell showing: c.j., cell junction; l, lysosome; m, microvilli; ps, pallial space; p.v., pinocytotic vesicles. Uranyl acetate stain. $\times 22,000$.



FIGURES 7-8.

between the bases of the microvilli arranged in linear arrays is a characteristic morphological feature of these cells (Fig. 6). These arrays terminate in the proximity of larger vacuoles or lysosomes. The lysosomes vary in size, are enclosed by a single structural membrane and often contain electron-dense particles (Fig. 7). Examination of selected areas of cells injected with colloidal gold demonstrates as shown in Figure 8 that ingested particulate matter comes to be localized in cell organelles we have identified as lysosomes.

DISCUSSION

Our study of the mantle epithelium of *Macrocallista* confirms the previous observations of Nakahara (1962) for *Pinctada* that the epithelium associated with shell formation ingests particulate matter derived from the pallial fluid. He indicated that the ingested material came to be localized in the Golgi region. Our observations show that the Golgi apparatus in these cells is widely distributed and accordingly Nakahara's observations in this regard are essentially correct.

Novikoff (1960) has listed several examples illustrating pynocytosis as the mechanism responsible for ingestion and intracellular transport of substances which do not readily permit passage through the cell membrane. We have identified pynocytotic vesicles indicative of cytotic activity and in addition have utilized colloidal gold, recognized in the electron microscope, to trace the pathway of ingested particles from the cell surface to lysosomes located in various parts of the cell. In a similar study on the segregation of ferritin by glomerular epithelia, it was shown (Farquhar and Palade, 1959), that ferritin particles accumulate first in pynocytotic vesicles, later in larger vesicles and finally in dense bodies or lysosomes. They assume that the same pathway is followed by other molecules, especially proteins of similar dimensions.

Our studies indicate that an apparently normal function of the mantle cells is the removal of particulate matter from the pallial fluid by means of pynocytotic activity. Studies currently in progress will attempt to show in more detail the nature of the materials removed by this method and also whether the removal of this material is associated with the mechanism of shell formation.

SUMMARY

1. Ingestion of particulate matter by the outer mantle fold of the calico clam, *Macrocallista maculata*, was studied. Following the introduction of carmine into the pallial space, dye particles were subsequently localized in the epithelia of the entire outer surface.

2. In an attempt to identify the cell structure in which the dye particles were localized, histochemical tests to identify acid phosphatase, lipids and mucopolysaccharides were employed. All of the above methods gave a positive reaction at the site corresponding to the locus in which the carmine was observed.

FIGURE 7. Electron micrograph of parts of two adjacent cells; uranyl acetate stain, $\times 13,000$. bm, basement membrane; c.j., cell junction; l, lysosomes; m, microvilli; mit, mitochondria; n, nucleus; p.v., pinocytotic vesicles; tegmental fiber designated by arrows.

FIGURE 8. Electron micrograph showing ingested particles of colloidal gold localized in lysosomes; $\times 73,000$.

3. Additional experiments were carried out in which colloidal gold was injected into the pallial fluid. Subsequent examination of epithelial cells showed that the colloidal gold was localized in organelles which, on the basis of fine-structure morphology and histochemical tests, we ascertain to be lysosomes.

4. Pinocytosis, occurring as a result of the pinching-off of the bases of the microvilli is a prominent activity of these cells. The micropinocytotic vesicles arising by this process apparently give rise to large vacuoles and lysosomes.

5. The intracellular mechanism by means of which ingestion by the mantle cells occurs has not previously been recorded. The significance of this activity awaits further study.

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ADAPTATIONS TO TEMPERATURE IN TWO CLOSELY RELATED STRAINS OF *EUGLENA GRACILIS*¹

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A general pattern has emerged from the many studies of physiological adaptations to temperature in protozoans. Within the limits of tolerance, low temperatures result in reduced growth rate and increased cell size, the latter a result of increased amounts of practically all biochemical constituents.

However, significant qualitative differences between species have also been reported. Thus, Johnson (1962) found that respiratory activity in the cryptomonad flagellate *Chilomonas paramecium* increased exponentially with temperature, while Buetow (1963) showed that respiration in a colorless mutant of *Euglena gracilis* var. *bacillaris* increased in a linear manner with temperature. The latter finding is of particular interest as an example of a continuously decreasing Q_{10} with increasing temperatures.

Qualitative differences of this sort must be an expression of genetic diversity among flagellates in a most fundamental aspect of protozoan physiology. Because of this, it seemed desirable to repeat Buetow's work with a wild-type *Euglena*. This report describes some physiological properties of two strains of *Euglena gracilis*, separated only by minor taxonomic differences, as a function of incubation temperature. These studies show marked quantitative but no qualitative differences in temperature adaptation between the two strains. Buetow's report of a linear increase in respiratory activity with elevated temperatures is confirmed when exogenous acetate is available, but a markedly different pattern was observed in endogenous respiration. Other parameters—mass, protein, and RNA—respond in the expected manner.

METHODS

Original stocks of the cells used, *Euglena gracilis* strain Z and *Euglena gracilis* var. *bacillaris*, were obtained from Dr. J. A. Gross and have been maintained through serial culture by the author. Growth rates of these stocks, measured under the same culture conditions, have remained constant over a period of years. For these studies, however, a single colony of each strain was picked off agar, inoculated into liquid media, and the resulting populations used. These two clonal populations, derived from single cells, did not differ in growth rate from the parent populations.

The salt medium of Cramer and Myers (1952), with sodium acetate (25 mM) as sole carbon and energy source, was used exclusively. Axenic cultures were

¹ Supported by Contract AT(04-1) GEN-12 between the Atomic Energy Commission and the University of California.

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grown in the dark in cotton-stoppered erlenmeyer flasks, maintained in water-jacketed incubators working against an ambient temperature of 10° C. Washed air was flushed continuously through the incubators.

Strain Z was examined at temperatures of 15°, 20°, 25°, 29°, and 34° C. *E. gracilis* var. *bacillaris* grew well enough at 15° C., but formed many clumps of cells (palmella) which made quantitative studies impossible at this temperature; this variety was studied at 17.5°, 20°, 25°, 29°, and 34° C. Temperatures above 32° are supra-optimal for *E. gracilis* and lead to irreversible bleaching. At each temperature, the cells were allowed to adapt through at least 15 generations before measurements were made. Population increase was followed by periodic cell counts with the Coulter cell counter. The cells were always harvested at a population density of 10⁵ cells per ml., well below levels of the stationary phase.

At most of these temperatures, cells were analyzed in terms of growth rate, mass, protein and RNA content, and respiratory rates, the latter both at the temperature of incubation and also at a test temperature of 25° C. For the respiration measurements, cells were harvested by gentle centrifugation (Buetow, 1961), washed three times with fresh culture medium (without acetate) and made up to volume in this wash medium. Oxygen consumption of the cell suspension was followed with the Beckman oxygen electrode, using a water-jacketed reaction vessel with constant stirring of the cells by a magnetic bar; with the electrode in position, this vessel was air-tight and contained no gas phase. Depletion of oxygen was recorded graphically, and absolute amounts of oxygen consumed calibrated against air- and nitrogen-flushed water. The temperature of the vessel was either 25° C. or the incubation temperature, held constant by circulating water from a refrigerated bath through the outer jacket of the reaction vessel. Endogenous respiration was followed for 20–30 minutes, after which acetate to 25 mM was added and respiration again followed for 20–30 minutes. Over this period of time, no extensive precautions against bacterial contamination were necessary. At the end of such a run, aliquots of the cell suspension were taken for cell counts, so that the respiratory rates could be referred to the average cell. Procedures for other measurements have been described previously (Cook, 1961).

RESULTS AND DISCUSSION

Growth rates

Generation times of the two cell types at the several temperatures are shown in Figure 1. At most temperatures, the Z strain of *Euglena* exhibits a faster multiplication rate than var. *bacillaris*. The degree of difference is not constant, however; at 29° C. (the optimum for both cells), the generation time of var. *bacillaris* is the greater by a factor of about 1.48; at 20° C. only 1.1. *E. gracilis* var. *bacillaris* does not respond as readily as strain Z to changes in temperature, at least in terms of multiplication rate. Figure 1 also shows that supra-optimal temperatures (34°) retard cell division more severely in the Z strain.

Cell mass

Figure 2 shows changes in total dry mass in cells adapted to the various temperatures. Between 20° and 29° C. var. *bacillaris* has the larger mass, but

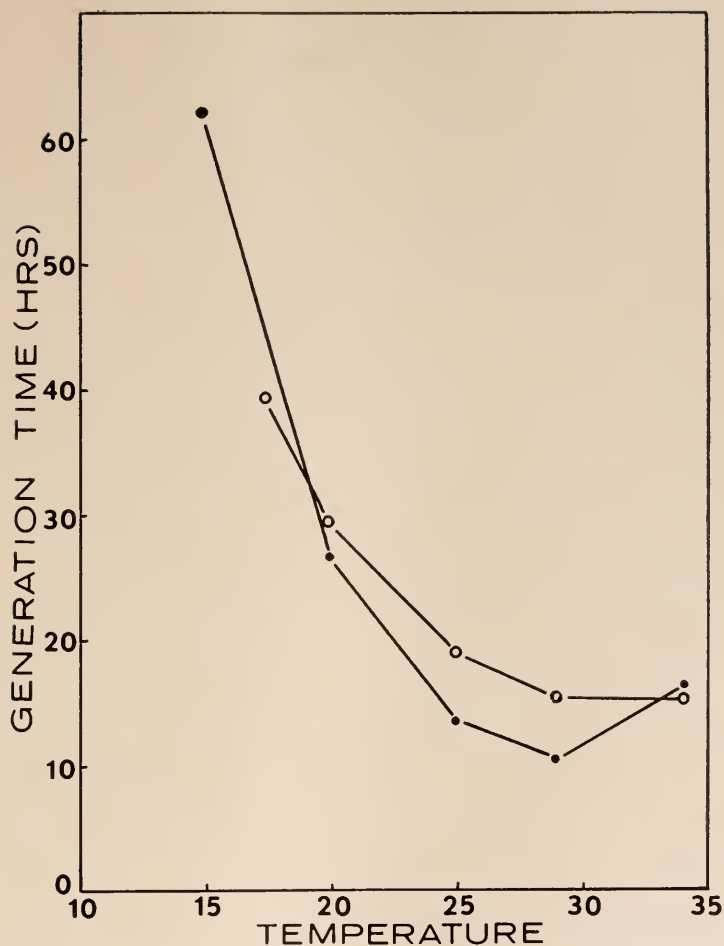


FIGURE 1. Generation times during logarithmic growth as a function of incubation temperature in *E. gracilis* strain Z (●) and var. *bacillaris* (○).

this condition is reversed below 20° C. Strain Z shows a minimum mass at 25°, but the change in var. *bacillaris* is essentially linear over the temperature range examined.

Protein and RNA

The protein content of these cells does not form a constant fraction of cellular mass. As the incubation temperature is changed, the protein fraction ranges between 20% to 30% of the total mass. However, the protein content in both cells changes in the same direction as total mass, being much increased at lower temperatures. Since the polysaccharide paramylum will make up most of the remaining mass, it is assumed that levels of paramylum must also change with temperature, and in the same direction as protein.

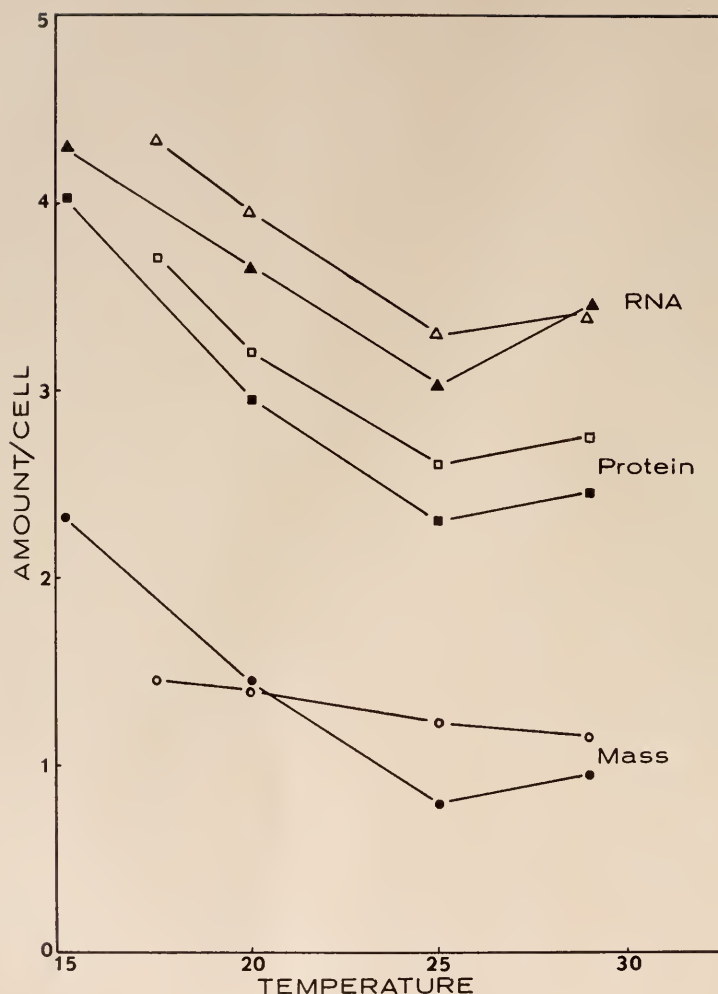


FIGURE 2. Average cell mass (circles), protein (squares), and RNA (triangles) in *E. gracilis* strain Z (filled figures) and var. *bacillaris* (open figures) during log growth at various temperatures. Ordinate values are in $\mu\mu$ grams and for the average cell should be multiplied by 10 (RNA), 100 (protein) and 1000 (total dry mass).

RNA levels roughly parallel protein content, being minimum in both strains at about 25° C. In *E. gracilis* var. *bacillaris*, the RNA content is equal to or slightly greater than that of the Z strain (Fig. 2).

Respiration

Figures 3 and 4 summarize respiratory characteristics. At the temperature of incubation, the respiratory rate in the presence of acetate is always greater in the Z strain, by an amount which is nearly constant at all temperatures (Fig. 3).

It may be noted from Figure 3 that these rates increase with temperature in a linear, rather than exponential, manner. In the absence of adaptive changes in respiratory machinery, an exponential pattern would be expected. Since this was not observed, it follows that the respiratory capacity of cells adapted to low temperature must be greater than those adapted to higher temperatures (Precht's type 1). That this is the case was demonstrated by the respiratory rates at a test temperature of 25° C. (Fig. 4). Cells incubated at the lower temperatures consume as much as 50% more oxygen at 25° than do those cultured at higher temperatures, in the presence of exogenous acetate.

Endogenous rates of respiration are essentially the same in both strains of *Euglena*. It is of interest to note that these rates do not change appreciably at temperatures below 25° C. when tested at the temperature of growth (Fig. 3). Endogenous respiration thus shows complete adaption to incubation temperature (Precht's type 2). There is a slight increase in the endogenous rate above 25°. Complete adaptation of this sort would also give rise to increased respiratory capacity at the lower temperatures, to an extent greater even than that found in the incomplete adaptation in the presence of exogenous substrate. That this is the case is seen in Figure 4. The endogenous consumption of oxygen of cells grown at 15–17.5° is about twice as great at 25° as that of cells grown at 25° C. It is concluded that the endogenous response of *Euglena* to temperature differs qualitatively from the response in the presence of exogenous acetate.

Rates of cellular processes

Rate constants for population expansion can be obtained from the generation times by use of the familiar growth equation, $k = \ln 2/\text{generation time}$. Synthetic

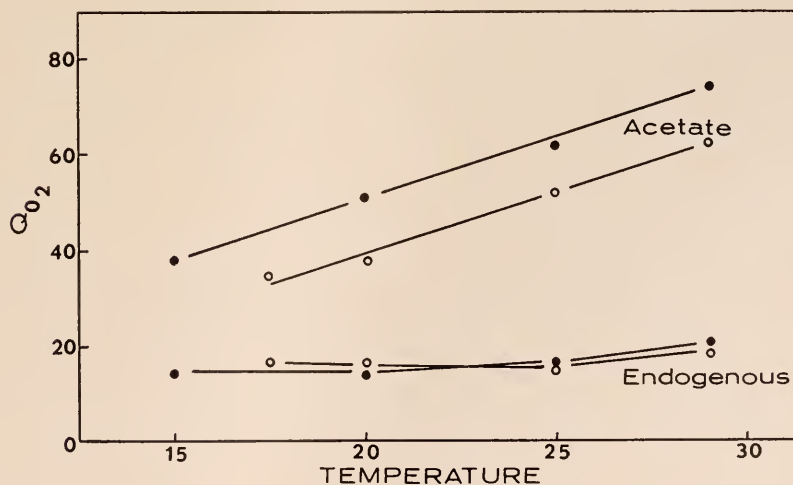


FIGURE 3. Oxygen consumption by *E. gracilis* strain Z (filled circles) and var. *bacillaris* (open circles) during log growth at the temperature of incubation. The lower curves show endogenous consumption, and the upper curves show consumption in the presence of exogenous substrate (acetate). $Q_{O_2} = \mu\text{l. O}_2/\text{hr.}/10^6$ cells.

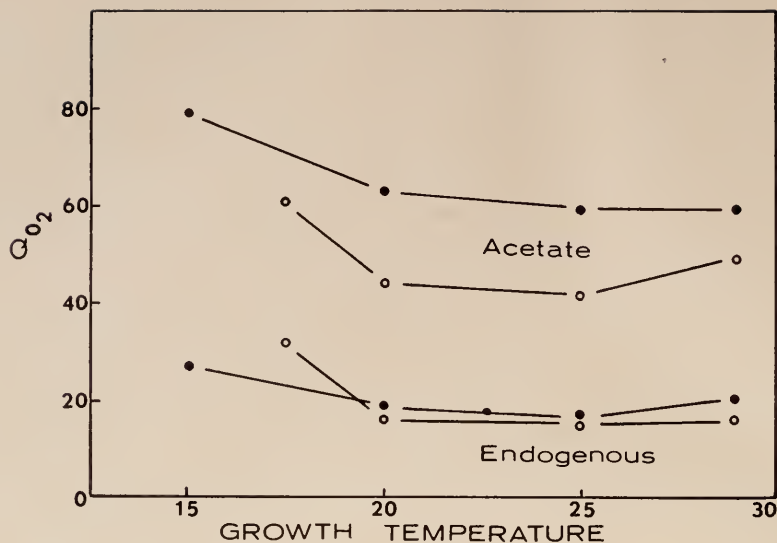


FIGURE 4. Rate of oxygen consumption at 25° C. by *E. gracilis* strain Z (solid circles) and var. *bacillaris* (open circles) after adaptation to growth at the temperature shown on the abscissa. Lower curves, endogenous rate; upper curves, rate in the presence of exogenous acetate. Q_{O_2} as in Figure 3.

rates can be estimated from

$$k = \frac{\bar{M}}{1.44 \text{ GT}}, \quad (1)$$

where k is the rate value (in amount synthesized per average cell per hour), GT is the generation time, and $\bar{M}$ is the amount held by the average cell in the constituent of interest (Cook and James, 1964). Synthetic rates for total mass, protein, and RNA for both strains of *Euglena* at the several different temperatures were calculated from this equation. Figure 5 is a logarithmic plot of these rates. The rate of mass accumulation by the average cell increases exponentially with temperature up to 29°; this rate increases rather more rapidly in the Z strain above 20° C. A more striking difference is seen in the rates of protein and RNA synthesis. While these rates increase exponentially with temperature in both cell types, the rate of increase in the Z strain is considerably greater than in var. *bacillaris*. In both strains, the rate of protein synthesis parallels the rate of RNA synthesis. At about 20°, the two cell types have equal rates of RNA and protein synthesis.

Q_{10} values

The Q_{10} values for these various processes can be read from the data shown in Figure 5. They are listed in Table I. The Q_{10} is approximately 2 between 15–17.5° and 29° C. for the rates of mass, protein, and RNA accumulation in var.

bacillaris and for mass accumulation in strain Z; the rates of protein and RNA synthesis in strain Z have Q_{10} values of 2.9 and 3.9, respectively.

The Q_{10} for division rate decreases from 3.3 to 1.3 over the range 17.5°–29° C. in var. *bacillaris* and drops to about 1 between 29° and 34° C. Strain Z is more sensitive to temperatures in terms of division rate, showing a progressive decrease in Q_{10} from 5.6 to 1.7 as the temperature is elevated from 15° to 29° C. (Table I). Above 29° C. the Q_{10} is less than unity in strain Z.

In the presence of exogenous acetate, the respiratory Q_{10} for strain Z is about

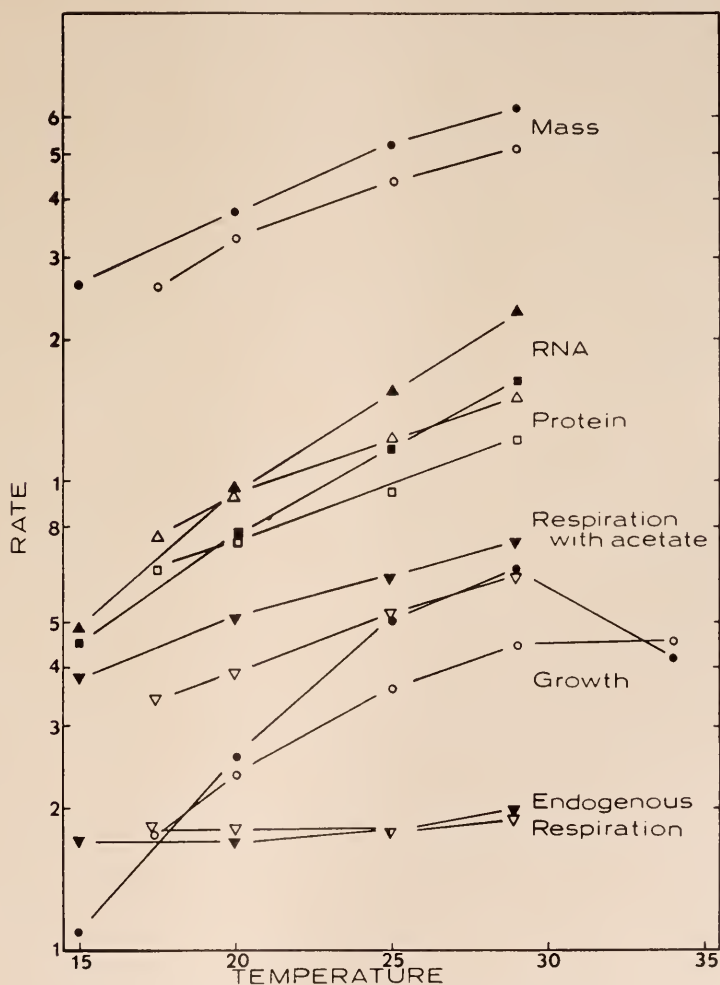


FIGURE 5. Log plot of metabolic and synthetic rates in *E. gracilis* var. *bacillaris* (open figures) and strain Z (filled figures). Rates calculated as described in text. Ordinate values for respiration ($\mu\text{l. O}_2/\text{hr.}/10^6$ cells, inverted triangles) are to be multiplied by 10; for growth rate (hr.^{-1} , circles) by 10^{-2} ; for RNA (triangles), protein (squares), and mass (circles), all in $\mu\text{gm.}/\text{cell}/\text{hr.}$, by 1, 10, and 10, respectively.

TABLE I
 Q_{10} values for various rates in two strains of *E. gracilis*

Temperature range	15(17.5)–20		20–25		25–29		29–34	
Cell strain	Z	b	Z	b	Z	b	Z	b
Growth rate	5.60	3.26	3.93	2.33	1.71	1.32	.41	1.05
Endogenous respiration	1.00	1.00	1.15	1.00	1.28	1.16		
Respiration (acetate)	1.84	1.73	1.49	1.73	1.49	1.61		
Mass increase	2.00	2.50	2.00	2.12	1.53	1.51		
Protein synthesis	2.90	1.74	2.35	1.74	2.35	1.74		
RNA synthesis	3.85	2.25	2.64	1.70	2.64	1.70		

1.5 between 20° and 29° C., and 1.8 between 15° and 20° C. Under comparable conditions, *E. gracilis* var. *bacillaris* shows an unchanging Q_{10} of 1.7 between 17.5° and 29° C.

It will be noted that respiratory rates at the temperature of incubation are shown on a linear scale in Figure 3, and on a semi-log plot in Figure 5. For *E. gracilis* var. *bacillaris*, a straight line satisfied the experimental points in both cases, which is merely to say that in a biological system it is difficult to demonstrate whether a function is linear or exponential when the range of values differs by no more than a factor of two. In the present case, the Q_{O_2} at 29° is 63 and at 17.5° it is 34.

The Q_{10} for endogenous respiration is about 1 in both strains at temperatures below 25°, and only slightly greater between 25°–29° (Table I). It was expected that respiratory activity in the presence of exogenous substrate (acetate) would be greater than the endogenous level, since acetate should stimulate activity of the Krebs' cycle enzymes. It was quite unexpected to find that the degree of stimulation was not constant as a function of temperature. The rate of oxygen consumption will be in part a function of the level of respiratory enzymes, excluding oxidative reactions which are not directly involved in respiration. That the activity of these enzymes is adaptively increased at lower temperatures is implied by the data for endogenous respiration shown in Figure 3. If the stimulatory role of exogenous acetate were strictly confined to respiratory activities, the Q_{O_2} with added acetate should be reflected in a Q_{10} nearly equal to that of the endogenous. That this is not the case is strong presumptive evidence for the view that oxygen is utilized in non-respiratory functions of *Euglena* cultured on acetate. Supporting evidence of a comparative nature is also suggested by the data shown in Figure 3: both strains of *Euglena* have essentially the same endogenous rate of oxygen consumption, but are stimulated to quite different levels of oxygen consumption by exogenous acetate.

Danforth and Wilson (1961) have shown that endogenous respiration of *Euglena* continues in the presence of exogenous substrate. The Q_{O_2} of *Euglena* adapted to growth and respiration on exogenous glucose is no greater than the endogenous Q_{O_2} , the latter having a level equal to the endogenous rate of acetate-grown cells (Cook and Heinrich, 1965). The optimal growth rate and the cellular mass and protein content are the same when cultured on either substrate. These

data taken together suggest that acetate-grown *Euglena* may consume oxygen *via* two principal routes: one as terminal electron acceptor in respiration, and the other in some non-respiratory and non-energy-yielding reaction(s) associated with growth on acetate.

Growth rate and synthetic rate

Schaechter *et al.* (1958) showed in the bacterium *Salmonella typhimurium* that mass and RNA levels were a positive exponential function of the growth rate but quite independent of temperature between 25° and 37° C., when the growth rate was varied by culture on different carbon sources. Since temperature affects growth rate as well as other physiological rates in *Euglena* with only acetate as carbon and energy source, it was of interest to know whether the observed effects were due primarily to temperature or indirectly to altered growth rates. The present data have been examined to determine whether the biochemical and physiological profiles of *Euglena* conform to some general pattern of the sort described by Schaechter *et al.* No such pattern was found. Figure 6 shows mass and RNA as a function

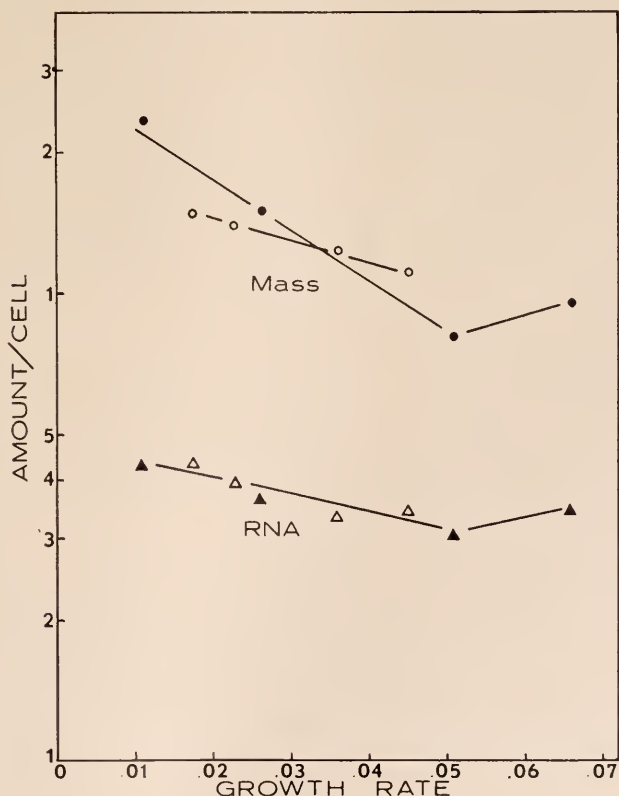


FIGURE 6. Log plot of mass and RNA content of *E. gracilis* strain Z (filled figures) and var. *bacillaris* (open figures) as a function of growth rate. Ordinate values for RNA are to be multiplied by 10 and those for mass by 1000 to give the amount in $\mu\text{g}/\text{m}$.

of growth rate, plotted for both *E. gracilis* var. *bacillaris* and strain Z. The data for protein are quite comparable (cf. Fig. 2) but are not shown in Figure 6 for purposes of clarity.

Mass and RNA levels in *Euglena* are a *negative* exponential function of the growth rate when the latter is .05/hr. or less, and a positive function at higher growth rates. In this respect it is noted that the growth rates of *S. typhimurium* as studied by Schaechter *et al.* (1958) were always considerably greater than those reported here for *Euglena*.

It is of interest to note that a single line satisfies the RNA content as a function of growth rate for both varieties of *E. gracilis*. In spite of the fact that strain Z

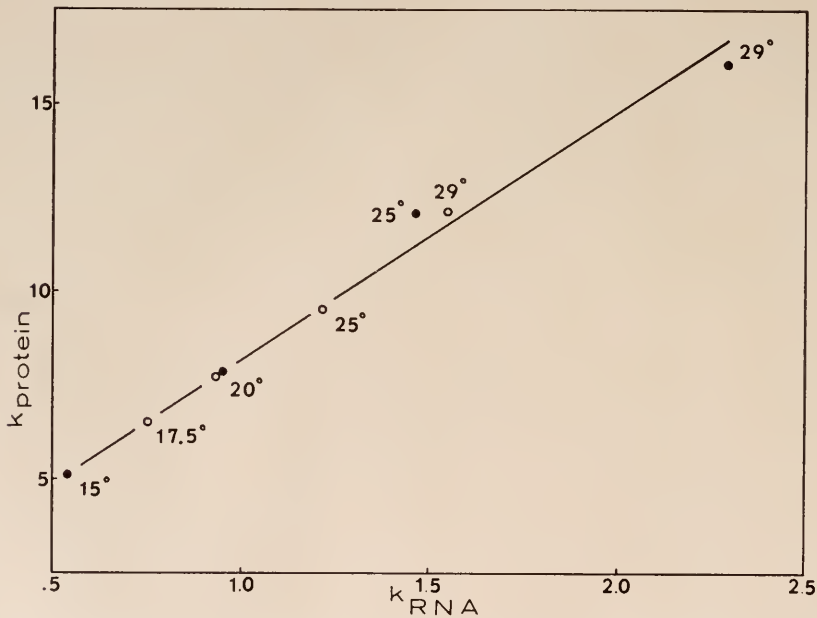


FIGURE 7. Relationship between the rates of RNA and protein synthesis in *E. gracilis* strain Z (filled circles) and var. *bacillaris* (open circles) at various temperatures as indicated. Rates in $\mu\text{gm./cell/hr.}$

and var. *bacillaris* can hold widely different values for RNA content and growth rate at a given temperature (cf. Figs. 1 and 2), the conformity shown in Figure 6 suggests that genetic differences in the two strains are not yet great enough to be expressed as some divergence in the fundamental relationships between division and synthesis. This view is strengthened by the comparison shown in Figure 7, which is a plot of the rate of RNA synthesis against the rate of protein synthesis for the two strains, when these rates are varied by temperature. Again, a single line satisfies both sets of data. The rate of information translation is the same in both cell lines, at least at a very gross level. By exclusion, it is inferred that the two strains may differ principally in the rate of information transcription. While DNA levels were not followed in the present study, it can safely be assumed that

the Q_{10} for the over-all rate of the DNA synthesis will be exactly equal to that of the growth rate. While DNA replication in the single cell is usually a discontinuous process, variable lengths of the S period could very well determine physiological and biochemical differences of the sort described in this paper, especially since DNA is presumed to be non-functional in RNA synthesis during its own replication (Prescott and Kimball, 1961). The possibility of ploidy is not excluded. It is suggested that the more sluggish behavior of *E. gracilis* var. *bacillaris* when compared to strain Z is the result in part of a slower rate of information transcription.

SUMMARY

1. Certain biochemical and physiological parameters in two closely related strains of *Euglena gracilis* (strain Z and var. *bacillaris*) have been examined after adaptation to various incubation temperatures.

2. The growth rate for the two strains differed at all temperatures, but was greatest in both at 29°.

3. Temperatures below optimal resulted in increased mass, protein, and RNA levels. In general *E. gracilis* var. *bacillaris* was larger in all these fractions at any given temperature.

4. Endogenous respiration proceeded at rates which were essentially unchanging over the temperature range 15°–29° C. Both strains exhibited the same rate.

5. Oxygen consumption in the presence of exogenous substrate (sodium acetate) increased in a linear fashion with the temperature of incubation, rates in strain Z being considerably greater than in var. *bacillaris*.

6. Mass, RNA, and protein content were found to be an exponential function of the growth rate, with a change in the sign of the slope at a growth rate of .05/hr. The rate of protein synthesis was a linear function of the rate of RNA synthesis in both strains.

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QUANTITATIVE ASPECTS OF BROWN ADIPOSE TISSUE THERMOGENESIS DURING AROUSAL FROM HIBERNATION¹

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Evidence which indicates that brown adipose tissue may serve as a specialized site of heat production has recently been reviewed by Joel (1965). The relative abundance of this tissue in hibernators has led a number of workers to suggest that brown adipose tissue may play an important role in the generation of heat during arousal from hibernation. There are, however, few data available which permit one to assess the quantitative aspects of such a role for this tissue. The recent attempts of Ball (1965) to calculate these quantitative aspects from available data in the literature, involved many assumptions which only served to emphasize the need for more data. This report describes one attempt to obtain such data for the big brown bat (*Eptesicus fuscus*) during its arousal from hibernation. The bat was chosen for this study because there is some evidence (Hayward *et al.*, 1965) to indicate that hibernating bats may be the most specialized of adult mammals in terms of the magnitude of brown adipose tissue thermogenesis.

A quantitative estimate of the contribution of heat by brown adipose tissue during an arousal from hibernation can be obtained by comparing the oxygen consumption of brown adipose tissue with the total body oxygen consumption during the arousal process. Ideally, the *in vivo* respiration of brown adipose tissue would be measured. However, the small size of the bats (about 15 grams) precludes such an approach. As an alternative, we have attempted to ascertain the heat production of brown adipose tissue from measurements of its *in vitro* respiration. The respiration of liver and heart slices from these bats has also been determined for comparison with brown adipose tissue respiration, and to obtain a larger estimate of the non-muscular heat production.

The limitations involved in efforts to summate *in vitro* tissue respirations to account for total animal respiration are well known (von Bertalanffy and Pirozynski, 1953). Such limitations have been invoked in this study to explain the failure of our tissue respiration measurements to provide a satisfactory estimate of the quantitative contribution of brown adipose tissue to arousal thermogenesis.

The technique of thermography has been used to provide additional descriptive evidence of the thermogenic capacity of brown adipose tissue.

¹ Supported by funds received from Life Insurance Medical Research Fund and U.S.P.H.S. grants A-3132, GM 05611-07 and GM 05197-08 and by Air Force Contract AF 31(609)-2296.

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METHODS

Prior to experimentation, the bats used in this study had been hibernating intermittently for a period of approximately five months. Measurement of their total oxygen consumption during arousal from hibernation was accomplished using a closed-circuit, volumetric respirometer. Exhaled carbon dioxide was absorbed by soda lime and the decrease in pressure in the closed system was detected with a sensitive manometer. At two-minute intervals, the pressure was restored to the initial level by allowing an accurately-measured quantity of water to flow into the system, this measure being equal to the oxygen consumed in that interval. The system was designed to maintain normal atmospheric gas concentration in the bat chamber and to minimize errors due to possible slight temperature fluctuations in the system.

To provide a criterion of stage of arousal from hibernation, each bat had, previously, a small thermocouple implanted in its interscapular brown adipose tissue. During an arousal in the respiration chamber the thermocouple lead wires were led from the chamber through an air-tight seal, enabling a continuous record of brown adipose tissue temperature to be obtained. All arousals were conducted at the ambient temperature at which the bats had been hibernating (5° C.).

Immediately subsequent to each arousal, the bat was sacrificed and a weighed sample of brown adipose tissue taken for the *in vitro* respiration measurements. The total remaining brown adipose tissue, from all locations in the body, was carefully dissected and weighed, care being taken to prevent drying of the tissue during this procedure. The heart and liver from each bat were also weighed and several samples of these tissues taken for respiration measurements.

Oxygen consumption of tissue samples was determined at 37.2° C. by means of the Warburg manometric apparatus. The incubation medium was Krebs-Henseleit phosphate buffer (Krebs and Henseleit, 1932) modified to contain one-half the recommended calcium. In some experiments, glucose was dissolved in this medium to yield a concentration of 1.5 mg./ml. The main compartment of the vessels contained 2.9 ml. of medium and the tissue sample. The center well contained 0.2 ml. of 20% KOH and a strip of filter paper to facilitate CO₂ absorption. The sidearm contained 0.1 ml. of a catecholamine solution dissolved in H₂O weakly acidified with HCl. The gas phase was oxygen. Brown adipose tissue was cut into small pieces with a razor blade after weighing to 0.1 mg. on a torsion balance, 15–20 mg. of tissue being used per vessel. Liver and heart were sliced with the aid of a Stadie-Riggs tissue slicer and weighed to the nearest mg. The amount of liver used per vessel was 100–175 mg. while heart samples weighed between 50–100 mg. In all cases, tissue samples were prepared and run immediately after removal from the bat. Vessels were gassed with oxygen for 10 minutes, closed, and after another five minutes for thermoequilibration, readings were taken at 10-minute intervals for 40–50 minutes. The contents of the sidearm were then added and readings continued for another 50–100 minutes.

The epinephrine used was a sample of the free base kindly supplied by Burroughs-Wellcome Co. The norepinephrine was a bitartrate preparation purchased from Calbiochem.

Bats were prepared for thermography (infrared radiography) by first shaving the hair off the dorsal aspect of their bodies. This facilitated the detection of distinct differences in the radiation of heat from the skin surfaces, these differences being largely dependent upon the temperatures of the underlying tissues. Thermo-

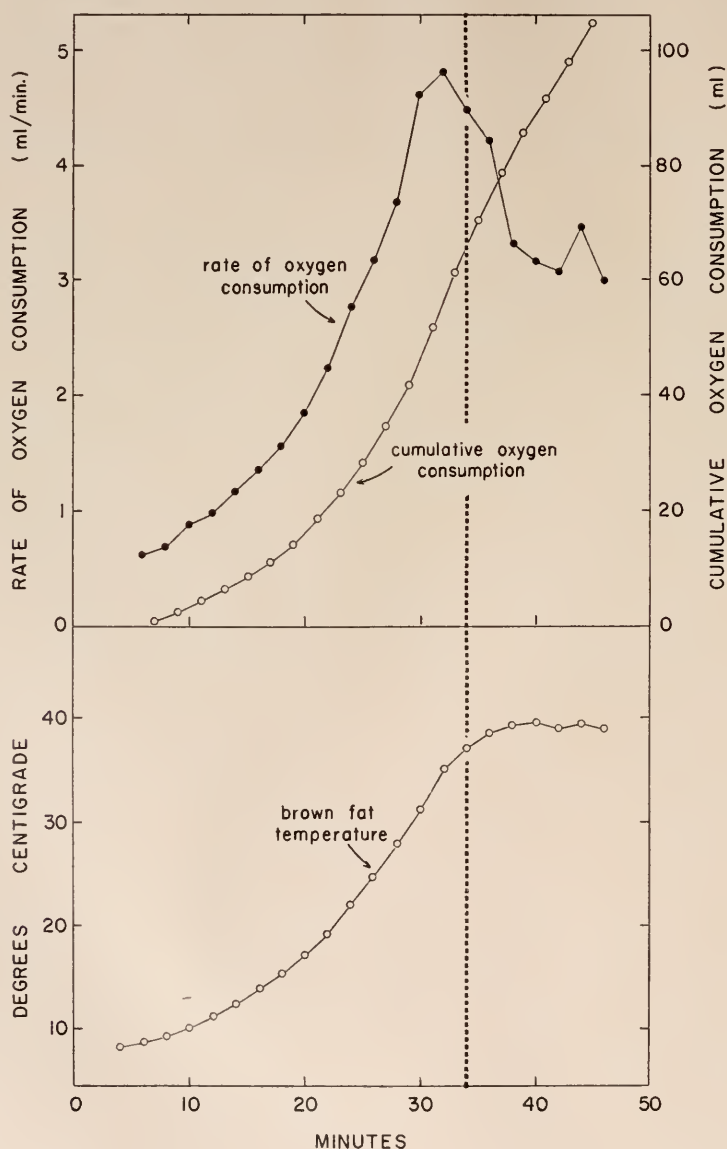


FIGURE 1. The typical patterns of oxygen consumption and brown adipose tissue (brown fat) temperature increase in the bat during arousal from hibernation. The vertical broken line intersects the curves at the time when rapid arousal is completed. Ambient temperature during the arousal was 5° C.

grams are pictorial thermal maps and were obtained using a Barnes Medical Thermograph.³

RESULTS

Total oxygen consumption during arousal

For each bat, the rate of arousal from hibernation varied to a certain extent with body weight, amount of brown adipose tissue, and apparent individual behavior differences. Amongst this variation, however, healthy individuals of similar body weight demonstrated a consistent pattern of oxygen consumption during arousal. The data of Figure 1 are an example of one such arousal. The brown adipose tissue (brown fat) temperature⁴ curve is included in Figure 1 to aid identification of the course of arousal. For approximately the first 15–20 minutes of arousal, there is a low and slowly-increasing rate of oxygen consumption, but

TABLE I
Rate of oxygen consumption of bat tissues at 37.2° C. under various experimental conditions

Tissue	Treatment	No. of animals	No. of measurements	Mean rate of O ₂ consumption $\pm$ S.E. (μ l./100 mg. fresh tissue/hr.)
Brown fat	Control	9	26	392 $\pm$ 42.4
	Epinephrine	9	9	1320 $\pm$ 93.9
	Epinephrine + glucose	6	6	1252 $\pm$ 107.7
Liver	Control	4	7	168 $\pm$ 5.0
	Epinephrine	3	3	160 $\pm$ 12.6
	Epinephrine + glucose	3	3	157 $\pm$ 9.3
Heart	Control	2	4	119 $\pm$ 11.6
	Epinephrine	2	2	117
	Epinephrine + glucose	2	2	114

the rate increases rapidly and reaches high levels for the 20–35-minute interval. After approximately 34 minutes (vertical broken line), the rate of oxygen consumption begins to fall to a lower level, coinciding with the approach of maximum brown adipose tissue temperature. At this point, the cumulative oxygen consumption is near 65 ml.

The mean figures for duration of arousal and total oxygen consumption for 8 bats are 36 minutes and 66.15 ml., respectively (from Table II). Assuming a low respiratory quotient (R.Q.) of 0.75 for arousal (Jansky and Hajek, 1961), this oxygen consumption represents 312 cal. of heat production, attributable to the enthalpy increase of the body mass and the heat loss during the arousal.

Tissue respiration

A representative experiment in which the oxygen consumptions of brown adipose tissue and liver were measured is shown in Figure 2. The respiratory rate of both

³ Barnes Engineering Company, Stamford, Connecticut.

⁴ The temperature of interscapular brown adipose tissue is typically about 1° C. above core temperature during arousal from hibernation (Hayward *et al.*, 1965).

TABLE II

Calculation of the percentage of the total oxygen consumption for arousal that is attributable to brown fat

Bat	Body weight (g.)	Duration of arousal (min.)	Weight of brown fat (g.)	Maximum metabolic rate of brown fat ($\mu\text{L O}_2/100 \text{ mg./hr.}$)	Total O_2 consumption for arousal (ml.)	O_2 consumption of brown fat for arousal (ml.)	Percentage of total O_2 consumption by brown fat
A	15.6	39	0.446	1500	78.05	4.35	5.57
B	14.2	39	0.453	1890	84.16	5.57	6.61
C	13.3	44	0.415	995	68.64	3.03	4.41
E	14.5	26	0.432	1490	51.88	2.79	5.38
G	14.8	22	0.584	1390	52.30	2.98	5.69
H	13.6	38	0.467	1420	71.33	4.20	5.89
J	15.6	38	0.643	1235	67.44	5.03	7.46
K	12.7	42	0.334	1070	55.39	2.50	4.52
Means	14.3	36	0.472	1374	66.15	3.81	5.69

tissues was unaltered by the addition of glucose to the medium. Addition of epinephrine, $1 \mu\text{g./ml.}$, caused a 500% increase in the rate of oxygen consumption of brown adipose tissue but was without effect upon liver. In other experiments in which the action of epinephrine on brown adipose tissue was compared at concentrations of 0.1, 1.0 and $10 \mu\text{g./ml.}$, it was found that a maximum response was obtained at $1.0 \mu\text{g./ml.}$ A comparison of norepinephrine and epinephrine at con-

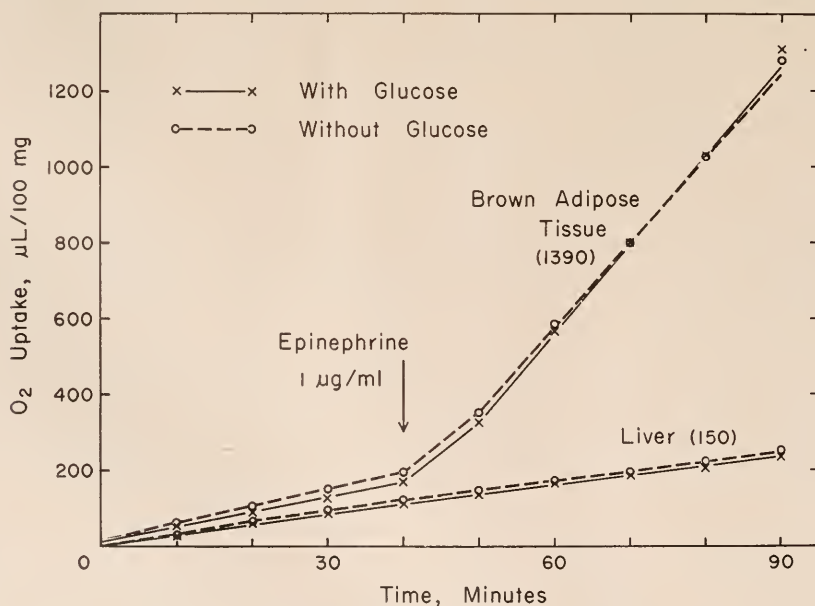


FIGURE 2. A representative experiment in which the oxygen consumptions of brown adipose tissue and liver of the bat show the effects of epinephrine and glucose addition. Numbers in parentheses are rates of oxygen consumption ($\mu\text{L}/100 \text{ mg. fresh tissue/hr.}$).

centrations of $0.1 \mu\text{g./ml.}$ also showed that no significant difference in their action could be observed. Epinephrine at a concentration of $1 \mu\text{g./ml.}$ was therefore employed routinely in the series of experiments performed.

A summary of this series of experiments is given in Table I. In the absence

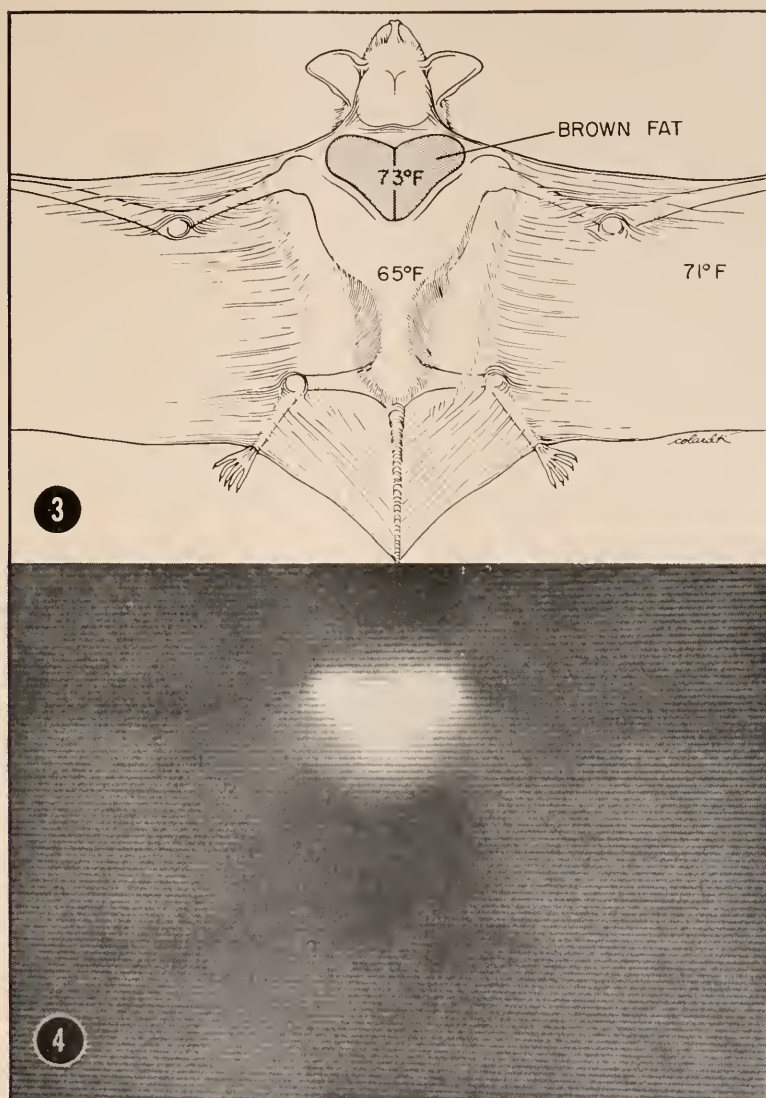


FIGURE 3. Illustration of a bat as positioned for thermography, showing the location of the interscapular brown adipose tissue and the major temperatures prevailing at the commencement of thermographic scanning.

FIGURE 4. Thermogram of the dorsal surface of a bat during its arousal from hibernation. The higher the temperature and intensity of infrared radiation from the skin surface, the brighter is the image.

of any added stimulation, the mean value for the rate of oxygen consumption of rate of brown adipose tissue and was without effect upon liver or heart slices. In experiments not reported here, it was found that there was no significant difference between the respiratory rate of brown adipose tissue sampled before and after arousal from hibernation.

brown adipose tissue was 2.33 and 3.29 times that of liver and heart, respectively. The addition of epinephrine caused an average increase of 350% in the respiratory

Relative oxygen consumption of brown adipose tissue

In Table II the pertinent data for calculating the percentage of the total oxygen consumption for arousal that is attributable to brown adipose tissue are summarized for each bat. Brown adipose tissue averaged 3.30% of the total body weight and, based upon its *in vitro* respiration, utilized an average of 5.69% of the total oxygen consumption. If the oxygen consumptions of liver and heart (averaging 4.31% and 1.16% of the total body weight, respectively) are included, a larger estimate of the non-muscular component of heat production is obtained. Together with brown adipose tissue, the mean oxygen consumption by these tissues for a 36-minute arousal would be 4.55 ml., or 6.88% of the total consumption.

Thermographic evidence of brown adipose tissue heat production

In the bat, brown adipose tissue is widely distributed between the muscles of the dorsal thoracic region, around the neck, and surrounding the major vessels entering and leaving the heart. The largest depot occurs in the interscapular fossa and extends to the back of the head and sides of the neck. The tissue lies just beneath the skin in the general position illustrated in Figure 3. To obtain the best contrast pattern on the thermogram, the following procedure was used. A hibernating bat was stimulated to arouse, and during this process was kept in a cold environment of 6° C. (42° F.) until the stage when the brown adipose tissue temperature was approaching the ambient room temperature of 22° C. (71° F.). It was then removed from the cold and held in the scanning field of the thermograph. The wing membranes rapidly equilibrated with room temperature. Within 2-3 minutes, the skin temperature over the brown fat passed room temperature, but the rest of the body was still cold. Infrared scanning was commenced when the major temperature areas were those shown in Figure 3. Scanning took approximately three minutes, during which time the bat had to be held immobile. The resulting thermogram (Fig. 4) shows a conspicuously-delineated "hot" area that coincides exactly with the shape of the underlying brown adipose tissue.

DISCUSSION

An average of only 6.9% of the total oxygen consumed by the whole bat during arousal from hibernation can be accounted for by measurements on brown adipose tissue, liver, and heart respiration *in vitro*. Even this value is high since the *in vitro* measurements were made only at 37° C., a temperature approached by the tissues only at the end of the arousal period. This is such a surprisingly low percentage of the total that it raises several questions.

First, if the data are accepted, one must still account for the remainder of the oxygen consumption. Muscle, because of its relatively large mass, would seem to be the main tissue to be considered. Rough dissections of muscle from several bats indicate the total muscle mass of an average bat to be about 4 grams. If, as our data would suggest, we attribute at least 75% of the total O_2 consumption during a 36-minute arousal period to muscle, then we obtain a rate of respiration for muscle of $\frac{0.75 \times 66.15 \text{ ml. } O_2}{4 \text{ g.} \times 36 \text{ min.}} = 0.345 \text{ ml. } O_2/\text{g. min.}$ The corresponding rate, as measured here, is 0.224 for brown adipose tissue and 0.02 for heart muscle. It is difficult to reconcile this very high rate of respiration for skeletal muscle with the indication that shivering is an unimportant feature of the arousal from hibernation of the bat, since the results of Hayward and Lyman (in press) show that there is no difference in the arousal time when bats are curarized. Moreover, if muscle was consuming oxygen at this rate, it should be producing over 50% more heat than brown adipose tissue. The thermogram presented certainly does not validate such a conclusion.

In considering possible reasons for this apparent discrepancy, the reliability of the measurements must be examined. For example, perhaps the measurement of total oxygen consumption is too high. For this measurement, however, all conceivable errors would result in underestimation rather than overestimation of the true rate. In addition, a theoretical calculation of the heat required to warm a 14.3-g. bat from 5° to 35° C. at an ambient temperature of 5° C. verifies the experimental results. If we assume an average specific heat of 0.9 cal./g.° C. for the total tissue of the bat, then $0.9 \text{ cal./g.}^\circ \text{C.} \times 14.3 \text{ g.} \times 30^\circ \text{C.} = 386 \text{ cal.}$ will be required for arousal. This value is undoubtedly too high since brown adipose tissue warms to 35° C. prior to all other tissues (Hayward *et al.*, 1965). Partly offsetting this consideration, however, will be the heat loss of the environment during arousal. A not unreasonable value would thus seem to be 300 ± 25 calories, which is comparable to the average of 312 calories from our experimental results. This calculation indicates that the measured total oxygen consumption is within the expected range and can be acquitted of possible major error.

Next, one may question the validity of the *in vitro* measurements of tissue oxygen consumption. The mean respiratory rate of 1374 $\mu\text{l. } O_2/100 \text{ mg./hr.}$ observed here for bat brown adipose tissue when stimulated by catecholamine is as high or higher than that reported for this tissue in other species. Joel (1965) gives an average value of 719 $\mu\text{l. } O_2/100 \text{ mg. fresh tissue/hr.}$ for brown adipose tissue from another hibernator, the ground squirrel (*Citellus tridecemlineatus*). This value was obtained in the presence of 1 $\mu\text{g./ml.}$ of norepinephrine and was raised to 1260 if 10 μmoles of succinate were also present. In the rat, a value of 725 was observed by Joel (1965) when the brown adipose tissue was stimulated by the addition of 1 $\mu\text{g./ml.}$ epinephrine. Smith and Roberts (1964) report values of 643 for brown adipose tissue from cold-acclimated rats and 232 from normal rats. These authors did not study the effect of catecholamine additions. The rates found here for heart and liver slices are not greatly different from those reported for rat tissues (Long, 1961, p. 795).

Lastly, one can offer the explanation that the measured *in vitro* rates fall far short of those that do occur *in vivo*. Certainly the measured rate for heart slices

does not reflect the rate exhibited by a heart actively beating *in vivo*. Values for perfused hearts are much higher (Fisher and Williamson, 1961). However, even if the *in vivo* rates of heart and liver respiration were 10-fold those measured here, they would still account for only 12% of the total oxygen consumption.

Dynamic metabolic conditions are characteristic of brown adipose tissue during arousal from hibernation (Joel, 1965, p. 84). We are led to suspect that brown adipose tissue heat production during arousal may be considerably greater than our respiratory data would indicate, despite their high value, and that the necessary conditions to achieve such rates *in vitro* have not yet been achieved.

We appreciate the critical advice given by Dr. C. P. Lyman on the design and conclusions of this study.

The use of the Barnes Thermograph was facilitated by the kind cooperation of the Department of Radiology, Massachusetts General Hospital, Boston.

SUMMARY

The *in vitro* respiratory rates of brown adipose, heart, and liver tissues were studied in the bat (*Eptesicus fuscus*) to determine their contribution to the heat necessary for arousal from hibernation. The mean oxygen consumption of the whole animal for arousal from hibernation was 66.2 ml. of which 5.7% is estimated to be utilized by brown adipose tissue, and 1.2% by heart and liver combined. The maximum respiratory rate of brown adipose tissue when stimulated by epinephrine was 134 μ l. O_2 /100 mg. fresh tissue/hr. Despite this high *in vitro* respiratory rate, it seems inadequate, on the basis of other evidence, to account for the heat production expected for brown adipose tissue during arousal from hibernation. A thermogram of a bat arousing from hibernation is presented which provides pictorial evidence of the large thermogenic capacity of brown adipose tissue. It is concluded that the conditions necessary to measure the maximum respiratory rate of brown adipose tissue, such as it occurs during arousal from hibernation, have not yet been achieved.

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MECHANISM OF STARFISH SPAWNING. I. DISTRIBUTION OF ACTIVE SUBSTANCE RESPONSIBLE FOR MATURATION OF OOCYTES AND SHEDDING OF GAMETES¹

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Since Chaet and his co-workers (Chaet and McConnaughy, 1959; Chaet and Musick, 1960; Chaet, 1964a) discovered that starfish (*Asterias forbesi*) can be induced to spawn by injecting a water extract of radial nerves into the coelomic cavity, some clue to the elucidation of the mechanism of starfish spawning has been afforded. The active substance responsible for gamete-shedding, contained in radial nerves, was reported to be a polypeptide with a relatively small molecular weight (Kanatani and Noumura, 1962, 1964; Chaet, 1964b, 1966). Further, the nerve extract prepared from one species acts similarly in several species, suggesting that the substances are chemically analogous among starfishes (Hartman and Chaet, 1962; Noumura and Kanatani, 1962; Chaet, 1964c, 1966), although there may be some species differences in details; for example, nerve extract of *Asterina pectinifera* is effective in inducing spawning of *Asterias amurensis*, but the converse is not true (Noumura and Kanatani, 1962; Chaet, 1966).

The present investigation was carried out in an attempt to determine the following points with regard to the distribution of active substance in the body of the starfish: (1) Whether or not there exists any difference in the quantity of active substance between male and female starfish, since males seem to shed their gametes more easily than females (Noumura and Kanatani, 1962). (2) Whether or not the active substance is present only in the radial nerves. (3) How the active substance is quantitatively distributed along the radial nerve. (4) Whether the active substance actually appears in the body fluid of the starfish at the time of natural spawning. An attempt is also made to discuss the mechanism of starfish spawning in relation to the results obtained from such investigations.

MATERIALS AND METHODS

The material mainly used was a common Japanese starfish, *Asterias amurensis*, during its spawning season. *Asterina pectinifera* was also used in some experiments dealing with comparative aspects. These starfishes were obtained from Tokyo Bay and kept in crawls hanging in the sea or in aquaria supplied with running sea water at the Misaki Marine Biological Station.

In order to obtain radial nerves, the ambulacral zone was isolated and then perpendicularly, but slightly diagonally, cut with scissors into two halves along

¹ Contribution No. 59 from The Ocean Research Institute, University of Tokyo.

the midline so that the nerve could easily be observed. These nerves were stripped off with fine forceps and collected. The nerves were stored frozen and dried by lyophilization. This method for obtaining the radial nerves seems to be more simple than the original method of Chaet (1964a).

For obtaining the nerve ring, a starfish was placed oral side up in a large Petri dish containing sea water, and the oral spines and tube feet near the mouth

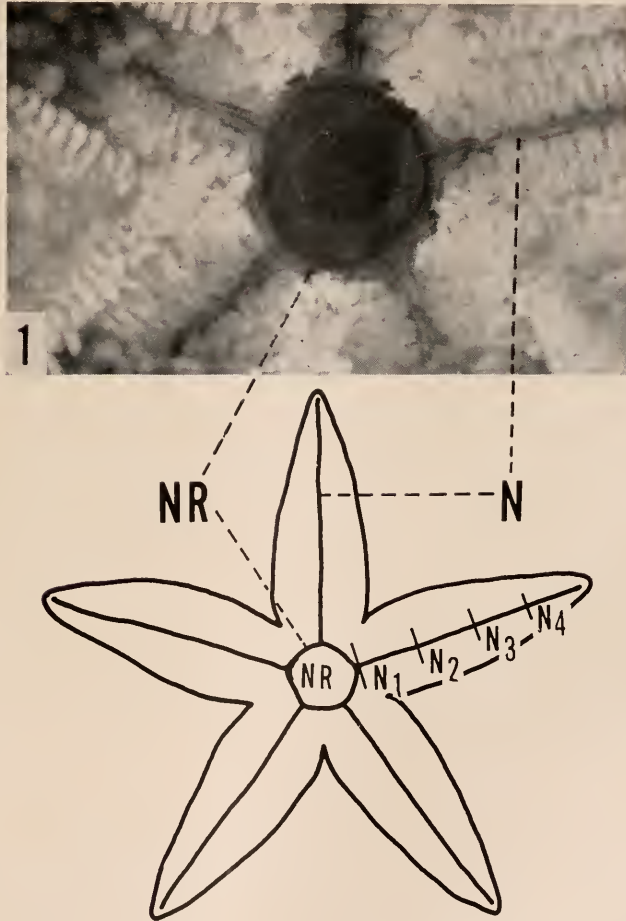


FIGURE 1. Nerve ring (NR) and radial nerve (N) of *Asterias amurensis*. Lower illustration shows parts of nerve used in experiment.

were carefully removed under a dissection microscope (Fig. 1). The nerve ring thus easily observed was cut out with fine scissors and lyophilized.

In an experiment comparing the activities of the various parts of radial nerves, the ambulacral plate was cut, after removing the nerve ring, into four sections as shown in Figure 1, and then the radial nerve of each part was stripped off as

described above and separately lyophilized. The parts of the radial nerves were designated as N_1 , N_2 , N_3 , and N_4 , from proximal to distal.

To make the nerve extract, a few milliliters of cold de-ionized water were added to the lyophilized material, which was then homogenized. To the homogenate, de-ionized water was added to give a concentration of 4 mg./ml. of lyophilized nerve and the mixture was centrifuged for one hour at 20,000 *g*. An equal volume of 1 *M* sodium chloride was added to the supernatant to make an isotonic nerve extract, which served as the original extract. In preparing test solutions, the original extract was diluted at various concentrations with sea water (successively diluted twice from 100 μ g./ml. to 0.8 μ g./ml., or from 200 μ g./ml. to 1.2 μ g./ml.). An extract of tube feet was made by the same procedure: the concentrations of the test solutions were adjusted from 500 μ g./ml. of lyophilized tube feet to 10 μ g./ml. by serial dilution with sea water. Extracts of body wall (ectoderm of the aboral surface of the arm), cardiac stomach and pyloric caeca were made by homogenizing the lyophilized samples in sea water. (The body wall was stripped off with fine forceps.) The homogenates were heated in a boiling water bath for five minutes and centrifuged. The supernatants were serially diluted with sea water. The concentrations with adjusted at 1000-32 μ g./ml. for body wall, 5000-312 μ g./ml. for cardiac stomach and 50-1.57 mg./ml. for pyloric caeca.

To assay the capacity of the test solutions to cause spawning and meiosis, an isolated small fragment of ovary, about 10 to 12 mm. in length, was placed in a small Petri dish containing 4 ml. of the solution and observed (Fig. 2) (*cf.* Chaet. Andrews and Smith, 1964). Ovarian fragments derived from a single female were used in each experiment in order to eliminate individual differences in reactivity. These ovarian fragments had previously been rinsed in sea water for an appropriate period until no more eggs were released from their cut surfaces. *In vivo* assays were also performed by injecting the test solution into the coelomic cavity of intact starfish.

To obtain coelomic fluid, a small slit was made in the aboral side of the distal part of an arm and the coelomic fluid was collected through it. The coelomic fluid thus obtained was used immediately after centrifugation for 30 minutes at 4000 r.p.m., or stored in a deep freezer before use.

RESULTS

Comparison of content of active substance between males and females

The radial nerves taken from each of four male and four female *Asterias* (arm length 10 cm.) were separately homogenized in 3 ml. of de-ionized water. The homogenate was diluted with an equal volume of 1 *M* sodium chloride, and 0.5 ml. of this nerve extract was injected into each of five starfish. As shown in Table I, the nerve extract obtained from male starfish was effective in inducing spawning of females, and *vice versa*. After shedding the gametes, the gonads were very small in most of these animals. Isolated ovaries placed in 50 ml. of sea water containing 2 ml. of these nerve extracts began to shed simultaneously (after about 30 minutes), regardless of the sex of the nerve donors. Control ovaries, placed in sea water containing 2 ml. of 0.5 *M* sodium chloride, failed to spawn. These preliminary experiments suggested that the spawning factor is the same in the two sexes.

Nerve extracts prepared from males were also effective in inducing meiosis of oocytes.

Experiments were next conducted to determine whether there exists any difference between male and female *Asterias* with respect to the content of shedding substance in the radial nerves, since the males shed in response to lower concentrations of injected nerve extract than do the females. Small fragments of the ovaries taken from a single female were exclusively used for assay in each experiment.

TABLE I

Induction of spawning by injected nerve extracts obtained from male and female Asterias

Groups of experiments	Nerve extracts obtained from					
	Male			Female		
	Sex	Time (min.)*	Amount of** gametes	Sex	Time (min.)	Amount of gametes
1	Male	29	+++	Female	39	+++
	Female	51	++	Female	39	+++
	Male	34	+++	Female	43	++
	Female	51	+++	Female	40	+++
	Male	34	++	Male	33	++
2	Male	26	+++	Male	26	+++
	Male	27	+++	Female	30	+++
	Male	28	+++	Female	32	+++
	Male	32	+++	Male	35	+++
	Female	40	++	Male	34	+++
3	Female	46	+	Male	28	++
	Male	33	++	Male	34	+++
	Male	No spawning***	—	Female	29	++
	Male	40	++	Female	37	+++
	Female	40	++	Female	37	++
4	Male	29	+++	Male	29	+++
	Female	35	+++	Male	29	+++
	Male	36	+++	Female	32	+++
	Female	No spawning***	—	Female	49	+
	Male	32	+++	Female	51	+++

* Interval preceding discharge of gametes.

** +++: large amount of gametes; ++: intermediate amount of gametes; +: small amount of gametes.

*** Gonads poorly developed, or had already spawned.

These ovarian fragments were placed in test solutions containing the original nerve extract at various concentrations (0.8–50 μ g. of lyophilized nerve per ml.), prepared separately from males and from females, and their spawning reactions were examined after one hour. Table II shows the results of six pairs of such experiments. These data clearly demonstrate that the content of shedding substance in radial nerves is the same in the two sexes.

TABLE II

In vitro assay to test for sex difference in gamete-shedding activity of nerve extracts.
Ovary fragments derived from one female were used in each pair of experiments

Concentration of nerve extracts ($\mu\text{g./ml.}$ lyophilized nerve)	Comparison of shedding induced by nerve extracts obtained from ♂ and ♀ donor starfish. A-F: ovarian fragments from 6 different females.											
	A		B		C		D		E		F	
	m.	f.*	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.
50	+	+	+	+	+	+	+	+	+	—	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+
12.5	+	+	+	+	+	+	+	+	+	+	+	+
6.3	+	+	+	+	+	+	+	+	+	+	+	+
3.1	—	—	—	—	+	+	+	+	—	—	+	+
1.6	—	—	—	—	+	+	—	—	—	—	—	—
0.8	—	—	—	—	—	—	—	—	—	—	—	—

+: positive, —: negative.

* Sex of nerve donor.

Quantitative distribution of active substance in various parts of radial nerve and in nerve ring

The results of *in vitro* assay of the active substance contained in the nerve ring and various regions of the radial nerves (Fig. 1) are shown in Table III. These data indicate that the active substance is present in the nerve ring as well as in the radial nerves, and is evenly distributed in quantity per dry weight among the nerve ring and the various parts of the radial nerves.

The absolute amount of active substance thus decreases from proximal to distal as the size of the cross-section of the radial nerve decreases (Table IV). The ratio of weights of lyophilized nerve at the various regions thus reflects the actual content of the active substance along the radial nerve.

In these *in vitro* experiments, the ovarian fragments began to shed after about 20–25 minutes (in some cases less than 20 minutes) when they were immersed in test solutions (4 ml.) containing more than 25 $\mu\text{g./ml.}$ of the lyophilized nerve.

TABLE III

Distribution of the gamete-shedding substance in nerve ring and radial nerves (Fig. 1) of Asterias

Minimum dose of nerve extract for induction of spawning within 1 hour in ovary fragments ($\mu\text{g./ml.}$ lyophilized nerve)						
Nerve donors	A (♂)	B (♂)	C (♂)	D (♀)	E (♀)	F (♀)
Parts of nerves						
NR	12.5	6.3	6.3	6.3	6.3	3.1, 6.3*
N ₁	12.5	6.3	6.3	6.3	6.3	3.1, 6.3*
N ₂	12.5	12.5	6.3	6.3	6.3	3.1, 6.3*
N ₃	6.3	6.3	6.3	6.3	6.3	3.1, 6.3*
N ₄	6.3	12.5	3.1	6.3	6.3	3.1, 3.1*

* Ovary from different animal.

In *in vivo* experiments, female starfish injected with 1 ml. of 0.5 *M* sodium chloride containing 500 μ g. of the same lyophilized nerve material usually began spawning after about 40 minutes.

Similar *in vitro* experiments on *Asterina pectinifera* clearly confirmed the above results obtained in *Asterias*; the minimum dose for induction of spawning was the same among the various regions of radial nerves and nerve ring (10 μ g./ml. of lyophilized nerve in the middle of June and 2.5 μ g./ml. in the end of June, respectively).

Presence of active substance in body parts other than central nervous system

Since nervous tissue is plentiful in the tube feet (Smith, 1937), the gamete-shedding activity of a tube-foot extract was next examined in a similar way. Table V shows the results of an *in vitro* assay, one hour after ovarian fragments were immersed in tube-foot extracts. These data show that the tube feet also contain shedding substance, and that there is little difference as regards its content between males and females. The minimum doses for induction of spawning were 50 to 100 μ g. of lyophilized tube feet per ml., when the experiments were conducted later in the spawning season. When such experiments were made earlier in the

TABLE IV
Weights of various parts of lyophilized Asterias nerves (Fig. 1)

Region	NR	N ₁	N ₂	N ₃	N ₄
Weight* (mg.)	2.0 $\pm$ 0.1**	9.0 $\pm$ 0.9	8.3 $\pm$ 1.5	6.7 $\pm$ 0.7	3.6 $\pm$ 0.3
Ratio	1	4.5	4.2	3.4	1.8

* Average of six animals (*ca.* 11 cm. arm length) shown in Table III.

** Standard deviation.

season, the minimum doses for induction of spawning ranged between 150 and 200 μ g./ml. Furthermore, breakdown of germinal vesicles had begun in most of the eggs discharged from ovarian fragments 30 minutes after immersion in test solutions containing 200 μ g. of lyophilized tube feet per ml. These data indicate that the tube feet actually contain the active substance, although the amount is small as compared with that in the radial nerves.

Some additional experiments were also carried out to determine whether the active substance exists in body regions other than the central nervous system and the tube feet. When the gamete-shedding activity of the body wall was tested *in vitro* with ovarian fragments, it was found that 125 μ g./ml. or more of the lyophilized material were effective. The extract of the cardiac stomach also showed shedding activity; 625 μ g./ml. or more were effective, whereas the extract of pyloric caeca had no effect even at a concentration of 25 mg./ml.

Occurrence of active substance in coelomic fluid at time of natural spawning

The injection of coelomic fluid, taken from an animal (*Asterias*) before or after spawning, into the coelomic cavity of another starfish with ripe gonads, failed to induce spawning.

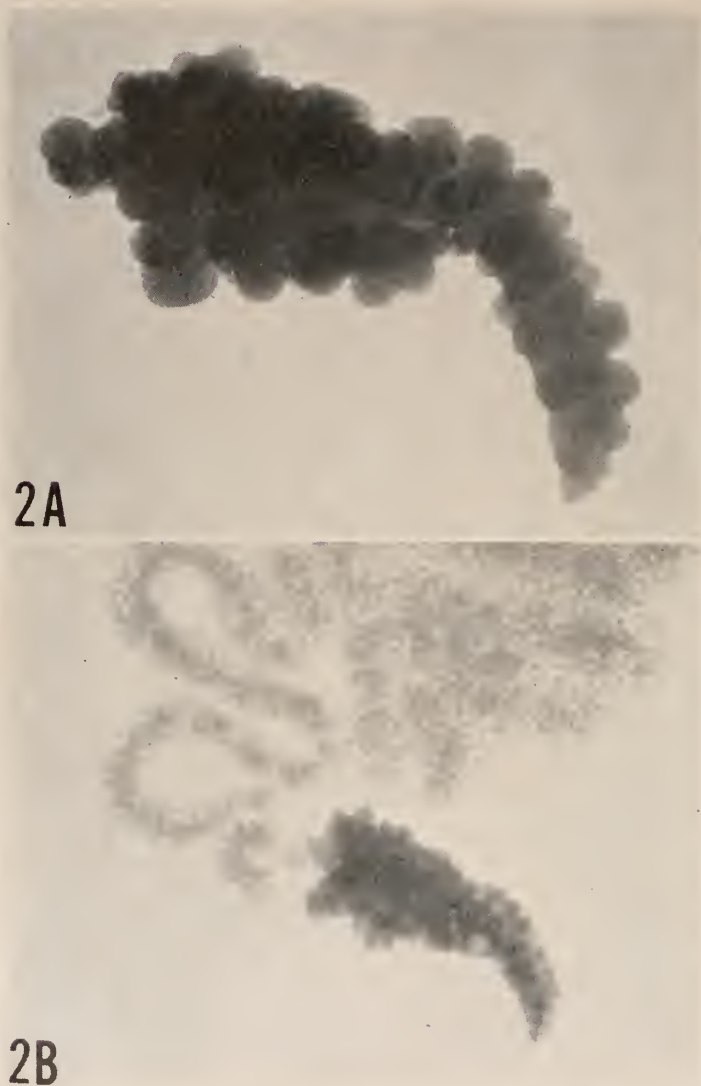


FIGURE 2. *In vitro* assay of gamete-shedding substance. A: ovarian fragment (ca. 12 mm.) of *Asterias amurensis*. B: same ovarian fragment discharging eggs after treatment with nerve extract. Same magnification.

However, coelomic fluid obtained from starfish at time of spawning showed gamete-shedding activity. When coelomic fluid was collected from a starfish spawning naturally in the laboratory and at once injected *in toto* into another starfish, spawning occurred in some cases.

In vitro experiments were next performed in order to verify more clearly the presence of the shedding substance in the coelomic fluid of naturally spawning starfish. Five to 20 ml. of coelomic fluid were separately collected from each of six

spawning animals (three males and three females). Small ovarian fragments were separately exposed to 2.5 ml. of such coelomic fluid. Controls were exposed to coelomic fluid from six other starfish which were not spawning.

The ovarian fragments immersed in the control coelomic fluid did not shed their eggs and the germinal vesicles of the ovarian eggs remained intact. On the contrary, spawning was induced within 15 to 20 minutes in all of the experimental fragments. The germinal vesicles in all the discharged eggs disappeared within 30 minutes after exposure of the fragments to the coelomic fluid. Observations after one hour revealed strongly shrunken ovarian fragments with thick alveolar walls, containing few, if any, eggs. These observations clearly indicate that the active substance is actually present in the coelomic fluid of a spawning starfish. When these test fluids were filtered and ovarian fragments taken from another starfish were immersed in them, shedding began after 14 to 27 minutes and the germinal vesicles underwent breakdown within 30 minutes. The degree of shedding reaction in these cases corresponded to that of the ovarian fragments exposed

TABLE V

In vitro assay of the gamete-shedding substance contained in tube feet of *Asterias*

Minimum amount of tube-foot extract necessary to induce spawning within 1 hour in ovarian fragments from 4 females									
Tube feet from males Exp. No.	μg./ml. lyophilized tube feet				Tube feet from females Exp. No.	μg./ml. lyophilized tube feet			
1	100,	100,	100,	50,	1	100,	100,	50,	100,
2	100	100	100	100	2	100	100	100	50
3	100	100	150	—	3	100	100	100	100
4	100	100	100	100	4	150	100	50	50
5	50	100	50	50	5	50	100	50	50
6	50	100	100	—	6	50	100	50	—

to the radial nerve extract containing 12.5 $\mu\text{g.}/\text{ml.}$ or more of lyophilized material. However, after the use of the same coelomic fluid twice in this way, the shedding activity decreased to some extent: ovary fragments in some experimental lots which were immersed for the third time began to shed only after 30 minutes and the spawning was incomplete. Germinal vesicles remained intact in some of the eggs within the ovary (10–20%). The shedding reaction in the third case seems to correspond to the case in which about 6 $\mu\text{g.}/\text{ml.}$ of lyophilized radial nerve were used. This reduction in inducing capacity suggests that the active substance is in some way taken up by the ovarian fragments, or that some inhibitory substance is released into the experimental coelomic fluid from the ovarian fragments.

DISCUSSION

According to Unger (1962), a substance which causes shedding of sperm is extractable from the radial nerves of male starfish only, in *Asterias glacialis*. The results of the present study, however, clearly demonstrate the presence of sperm-shedding substance in the radial nerves of female as well as of male *Asterias*

amurensis (Table I). The so-called egg-shedding substance was also obtained from male starfish; moreover, *in vitro* experiments using isolated ovary fragments showed that the content of the active substance in the radial nerves is equal in the two sexes. Therefore, the substance responsible for inducing the release of sperm is believed to be identical with the substance which induces the release of eggs. The active substance was also found to be equally effective in inducing spawning in both sexes at several steps in the course of chemical purification, and could not be separated into two different substances specific to males or females (Kanatani and Nourmura, 1964; Kanatani, unpublished). Chaet (1966) has recently reported that the shedding substance is present in *Asterias forbesi* nerves of both sexes, and in the same concentration. According to his experiments using intact starfish, shedding substance taken from nerves of either sex stimulated shedding in both sexes.

With respect to the location of the gamete-shedding substance in a single starfish, Chaet (1966) reports that hot (76° C.) salt water extracts prepared from various tissues other than radial nerve of *Asterias forbesi*, including tube feet and the oral and aboral surfaces, show no gamete-shedding activity. It is quite possible that his extraction procedure was inadequate in this case. As is clearly shown in our present results, the gamete-shedding substance is not confined to the nerve ring and radial nerve. For example, extracts of tube feet, body wall and cardiac stomach also showed the shedding activity. Considering the order of the shedding activity expressed by the extracts of various regions of the body on the one hand, and the results of the extensive study of Smith (1937) on the distribution of the nervous system of starfish on the other hand, the quantitative distribution of the active substance in the starfish seems to correspond to the quantity of nervous tissue present in a given part of the body. Within the radial nerve, Uter (1966) has reported that the active gamete-shedding substance is located only in the most aboral region of the nerve.

Chaet (1964c, 1966) has suggested that the radial nerves also contain a substance, "shedhibin," which inhibits the action of the gamete-shedding substance. According to his opinion, natural control of shedding is regulated both by the level of shedding substance and the presence (or absence) of shedhibin. The nature of shedhibin, however, is still obscure. In this connection, attention must be drawn to the observation made in the present study, that the gamete-shedding substance is present in the coelomic fluid only at the time of spawning, even during the breeding season. That the shedding substance can act directly on the gonads has been clearly demonstrated in our previous work, in which the gamete-shedding substance was locally applied to an isolated ovary (Kanatani, 1964). The ripe, distended gonads, therefore, will readily respond to the shedding substance when it is released into the coelomic cavity. It thus appears highly probably that spawning is controlled by some mechanism which introduces the gamete-shedding substance into the coelomic cavity in which the gonads are suspended. The problems remaining to be solved are: (1) from what part of the nervous system and through what route the shedding substance reaches the coelomic cavity; and (2) by what means such transport of the substance is controlled.

It is unfortunately difficult to determine the actual amount of gamete-shedding substance released into the coelomic cavity, because the substance, when released into the cavity, seems to be readily absorbed by the gonads which occupy most of

the space and present an extremely large surface area because of their complicated branching structure. *In vitro* experiments on the shedding activity of coelomic fluid carried out in the present study strongly suggest that the substance is actually taken up by the ovarian fragments. Evidence that *Asterias* gonads can absorb labeled amino acids and glucose from dilute solution in sea water and coelomic fluid (Ferguson, 1964b) supports this suggestion.

In the foregoing considerations the gamete-shedding substance is thought of as being transported first into the coelomic cavity and acting directly, from the outside, upon the gonads. However, it is also possible that the shedding substance responsible for natural spawning is released directly into the gonad, either from the genital sinus *via* the aboral coelomic sinus or from the nerves distributed in the gonad, with an excess of the substance diffusing into the coelomic fluid through the coelomic epithelium. Against this possibility, histological observation of the ripe, distended ovary shows that the genital sinus is so strongly compressed as to be hardly discernible. Moreover, the strictly localized effect of externally applied nerve extract (Kanatani, 1964) argues against the existence of an internal transport system within the ovary. Finally, the gonads are less well innervated than other organs. On the other hand, shrinkage of the gonads takes place in the same way after natural spawning as after spawning induced by the presence of nerve extract in the coelomic cavity. Moreover, the recent investigations of Ferguson (1964a, 1964b) have demonstrated that the starfish coelomic fluid plays an important role in the transport and exchange of some substances between tissues, regardless of whether they are located in the coelomic cavity or in some other space.

It is therefore considered highly probable that at the time of spawning the shedding substance is released into the coelomic fluid and taken up by the gonads, where it acts to bring about meiosis of oocytes and spawning of the gametes.

Although it is known that the gonad becomes more sensitive toward the end of the spawning season, it has not yet been determined whether the shedding substance is abruptly released into the coelomic cavity at each spawning or accumulates little by little until a threshold concentration is reached.

We wish to express our gratitude to Dr. J. C. Dan for her encouragement and advice, and to Misses K. Fujino and H. Shirai for their technical assistance. Our thanks are due to the director and the staff of the Misaki Marine Biological Station for putting the research facilities of the station at our disposal.

SUMMARY

1. The localization in the starfish body of the active substance responsible for maturation and gamete-shedding was determined by *in vitro* assay, using *Asterias amurensis* and *Asterina pectinifera*.

2. The active substance was found in the radial nerves at the same concentration in both male and female starfish, suggesting that the testis responds more readily to the action of the substance than does the ovary. The gamete-shedding substance seems to be identical in the two sexes.

3. As determined by the shedding reaction of isolated fragments of ovaries, the quantity of active substance (per dry weight of lyophilized nerve materials) was found to be uniform in various parts of the nerve ring-radial nerve system.

4. Tube feet and the body wall also contained considerable amounts of the active substance, although the content was several times lower than that of the radial nerves.

5. The shedding activity was also detectable in the extracts of some other regions of the starfish in which nervous tissue is plentiful: for example, cardiac stomach. However, the activity was much less than that of the radial nerves.

6. The active substance was found in the coelomic fluid only when the starfish were undergoing natural spawning. The coelomic fluid of starfish which were not spawning did not show any shedding activity, regardless of the condition of their gonads.

7. The significance of the appearance of active substance in the coelomic fluid in relation to the mechanism of starfish spawning is discussed.

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AN ENDOGENOUS DIURNAL RHYTHM OF BIOLUMINESCENCE IN A NATURAL POPULATION OF DINOFLAGELLATES¹

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Several authors have shown or suggested that dinoflagellates are the major source of bioluminescence in many surface regions of the ocean (Backus, Clark and Wing, 1965; Backus, Yentsch and Wing, 1961; Gold, 1965; Hardy and Kay, 1964; Seliger *et al.*, 1961, 1962; Sweeney, 1963; Yentsch, Backus and Wing, 1964; earlier work summarized by Harvey, 1952, p. 124). Hastings and Sweeney (1957, 1958) and Sweeney and Hastings (1957) have studied an endogenous diurnal rhythm of light production in laboratory cultures of the dinoflagellate *Gonyaulax polyedra*. Earlier work summarized by Harvey (1952, p. 128) has suggested an endogenous rhythm in flashing, but lack of dark-adaptation of the observer makes these reports questionable. Harvey (1952, p. 129) reports a more careful experiment but in an abnormally eutrophic environment. None of these reports give quantitative measurements, and none of these compare the endogenous influences with the exogenous influence of light inhibition.

An *in situ* diurnal rhythm of luminescence within the euphotic zone, probably caused by dinoflagellates, has been found by Backus *et al.* (1961) and Clarke and Kelly (1965), although this rhythm has not been shown to be endogenous. Other workers have found an *in situ* rhythm and concluded that it was exogenous in origin. Seliger *et al.* (1961, 1962) postulated that the rhythm was controlled by a diurnal migration of the luminescent dinoflagellates. Yentsch *et al.* (1964) pointed out that photo-enhancement and photo-inhibition alone might explain the amount of bioluminescence and that diurnal migration was not involved. Backus *et al.* (1965) found that bioluminescent organisms in Eel Pond responded to the eclipsing sun much as they normally respond to the setting sun, and that their behavior from mid-eclipse to eclipse end resembled dawn behavior. They concluded that the exogenous factor of changing light overrides such endogenous rhythms as may exist.

The purpose of the work reported here was to resolve the relative importance of endogenous and exogenous influences on the diurnal rhythm of bioluminescence of a natural population of phytoplankton under controlled conditions, and to identify the members of the population responsible for the luminescence in a typical inshore marine environment.

METHODS

Surface water was taken at various times of day from near the entrance to Eel Pond—a salt pond in Woods Hole, Mass., which is tidally flushed by water from

¹ Contribution No. 1745 from Woods Hole Oceanographic Institution. Research supported by National Science Foundation Grant 2435.

the connecting harbor, and which has phytoplankton populations similar to those in the harbor. This water was filtered through 0.33-mm. aperture netting, and placed in a Teflon-lined 15-gallon steel drum. The contained organisms were stimulated by controlled air flow from an aquarium bubbler "stone" placed near the bottom, and bioluminescence was measured using a photomultiplier photometer with logarithmic output and sensitive to intensities as low as 10^{-8} $\mu\text{W.}/\text{cm.}^2$. The photometer window was in the water 8 cm. above the bubbler, and the output was recorded on a Sanborn strip-chart recorder with 0.01 second response time. Organisms were stimulated for 40 to 60 seconds at various times depending on the particular experiment and flashing was recorded during stimulation. Total flashes were counted for the first 30 seconds of stimulation, and bioluminescence expressed as flashes/30 seconds. This measurement was used rather than total light output since amount of flashing is an ecologically more meaningful quantity, and since it was impossible to know the number of organisms subject to stimulation. Stimulation provided sufficient mixing to prevent stratification of the organisms. All experiments were performed in a darkroom at temperatures between 20° and 22° C.

EXPERIMENTS

Three types of experiments were performed. The first measured the endogenous luminescence rhythm by recording luminescence of populations kept continually in darkness. The second studied the recovery of ability to luminesce when populations taken from normal daylight in the natural environment were placed in darkness. The third group of experiments measured the effects of exposure to light at various times of day on the luminescence of populations kept in darkness.

In the first experiment, water was collected, filtered, and placed in complete darkness in the laboratory just prior to 1900, 16 Aug., 1965, and stimulated flashing was recorded every hour from 1900 until 0300, 20 Aug., 1965. Flashes/30 sec. are plotted against time in Figure 1. An endogenous rhythm of flashing rate was apparent and continued for three days, although the maximum flashing rate was lower each day. A similar experiment was performed between 3 Aug. and 5 Aug., 1965, and although the recording methods were different, the results were qualitatively the same. These results are qualitatively similar to those found by Sweeney and Hastings (1957) who measured total light output by cultures of *Gonyaulax polyedra*. The changes in flashing rate are also similar to *in situ* measurements made by Backus *et al.* (1961) except that the morning decrease and evening increase in flashing are not as pronounced in the present work.

In order to study recovery from inhibition due to daylight, two series of experiments were performed in which water was brought from the surface of Eel Pond into complete darkness at various times of day (daylight intensities from 5×10^4 to 1×10^5 $\mu\text{W.}/\text{cm.}^2$, measured with a General Electric photoelectric meter). Flashing rates were recorded every hour thereafter until 2300. The two series gave similar results, and the results of the second series and the times of start of dark exposure are shown in Figure 2. Rates of flashing throughout the day of organisms in continuous darkness are shown for comparison (results of Aug. 30 experiments; see below and Figure 3).

Flashing rates in water collected during daylight increased within two hours to the rate shown by a population kept in darkness for the previous night, and

then followed the curve for that population. Flashing rates in water taken at night were initially much higher. Thus, inhibition of flashing by daylight superimposes its effect upon a daytime decrease controlled by an endogenous rhythm. This is further emphasized in the next group of experiments.

The third group of experiments examined the effects of inhibition by exposure to short periods of artificial light at various times of day. On three occasions water was brought into the darkroom at dusk (2000) and the included organisms were allowed to dark-adapt until midnight. They were then exposed to 15 minutes of light every two hours for 24 hours and luminescence was recorded 15, 30, 45, 60, 90 and 120 minutes after start of light exposure. Intensities at the surface of the water, dates, and certain minor departures from the described schedule are shown in Figure 3. The lower surface light intensity at $1470 \mu\text{w./cm.}^2$ was provided by

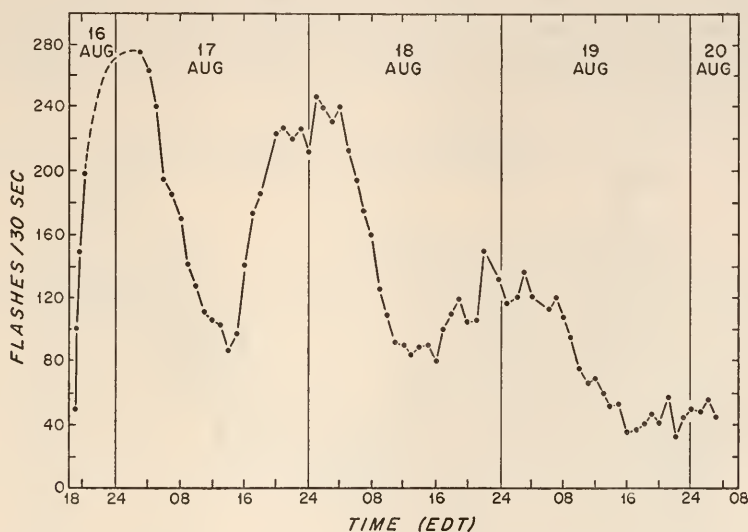


FIGURE 1. Flashing rates during stimulation for 30- to 60-second duration recorded in water collected at 1900 hr. 16 Aug. 1965 and kept continuously in the dark for the period shown.

placing over the barrel a bank of fluorescent lamps, rated by the manufacturer to have a spectral distribution similar to normal daylight. The higher light intensity of $8820 \mu\text{w./cm.}^2$ was provided by an incandescent spotlight which had a different spectral distribution and angular dispersion. Light intensity was attenuated by about 30% through a 60-cm. water layer in Eel Pond, and probably by a similar amount in the barrel. The rising air bubbles used for stimulation mixed the water and assured random dispersal of the organisms with uniform exposure to light.

Results of these experiments are shown in Figure 3. The lower curves connect the flashing rates after 15 minutes of light exposure, and the upper the rates after active recovery from light inhibition had apparently stopped (1 hr. 45 min. after exposure). Although the figures differ somewhat, presumably because of population changes, they are all similar in that they show proportionately greater inhibition

during daylight hours. Since the treatment and environmental conditions were the same both day and night, it may be concluded that there is an endogenous diurnal rhythm in sensitivity to light inhibition. The similarity of the flashing rates after recovery from light inhibition to those of populations kept in continuous darkness indicates that there is no appreciable long-term effect of light exposure.

The two intensities used are approximately equivalent to 2% and 12% of the mid-day surface light intensity in Eel Pond. Although the higher intensity of 8820 $\mu\text{w.}/\text{cm.}^2$ caused slightly greater inhibition, the flashing was never reduced by more than $\frac{3}{5}$. Sweeney, Haxo and Hastings (1959) noted that exposure of *G. polyedra* cultures to light caused inhibition of luminescence to varying degrees, depending on the intensity of the light, and that longer exposure to light altered the phase of the rhythmicity. They did not, however, mention significant variations in sensitivity to inhibition with time of day. The lack of a phase shift in the present experiments was probably due to the relatively short exposure and low intensity. Many luminescent marine organisms are known to be inhibited by light (Harvey, 1952), but only dinoflagellates and euphausiids (Mauchline, 1960) are known to have

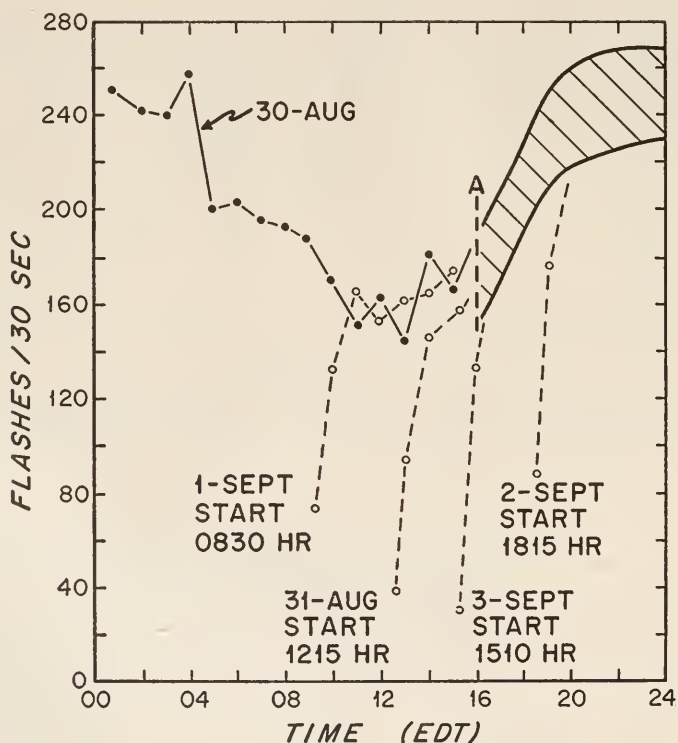


FIGURE 2. Flashing rates recorded using water collected from the natural environment at dates and times shown, and placed immediately in darkness. After time "A" (1600 hr.), all flashing rates fell on approximately the same curve, and only the range of flashing rates is shown by the cross-hatched area.

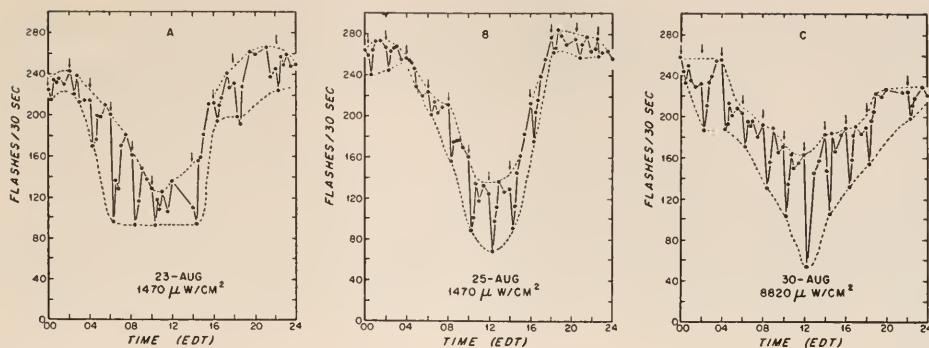


FIGURE 3. Effect of light inhibition at various times of day. Dates, times, and light intensities as shown. Times of start of light exposure for 15 minutes are indicated by arrows. Upper dashed line connects rates after complete recovery; lower line connects rates after light exposure.

an endogenous rhythm in flashing activity, and there are no reports known to us of an endogenous variation in sensitivity to light inhibition.

DISCUSSION

These experiments with natural populations brought into the laboratory attempt to bridge the gap between the studies of luminescence in cultured dinoflagellates made by Hastings, Sweeney, and co-workers, and the previous field studies in which rhythms in dinoflagellate luminescence were found (references in the introduction). Our results indicate that the flashing rates of populations kept in darkness decrease during daytime hours, and that the effect of light in causing inhibition of flashing is greater during daytime. Both the dark-adapted flashing rates and the sensitivity to photo-inhibition are controlled by an endogenous diurnal rhythm.

Hastings and Sweeney (1958) found an endogenous rhythm in the effect of periods of light exposure on changing the phase of the luminescence rhythm. Their effect had a maximum sensitivity during dark hours in contrast to the varying sensitivity to light inhibition found here, which has a maximum during daylight. It may be inferred from this that unless the experimental organisms vary, different mechanisms are involved in these two manifestations of light sensitivity.

Hastings and Sweeney (1958) also found a greater night-day variation in light production than is found here. This is probably because they measured total light output rather than number of flashes. Since in their experiments the intensity as well as rate of flashing was greater at night, total light output increased to a greater extent. This night-time increase in intensity was not apparent in our records.

Because the light-inhibition effect is the most obvious with *in situ* measurements, several of the authors mentioned in the introduction have considered control of flashing to be only exogenous, but this is apparently an oversimplification. Yentsch *et al.* (1964) found that a model involving only photo-enhancement and photo-inhibition described the diurnal variation, but it appears that this is useful only as an empirical approximation. Seliger *et al.* (1961, 1962) have hypothesized diurnal

migration as the cause of variation in light production, but this appears to be neither sufficient nor necessary to explain the variations we observed.

Backus *et al.* (1965) have described the effect of a solar eclipse on luminescent activity to be similar to that of the setting sun, and they concluded that the exogenous factor of changing light overrides such endogenous rhythms as may exist. Experiments described above, however, showed that populations brought into darkness from complete daylight in the natural environment increased their flashing rate only by an amount determined by the diurnal rhythm and not to a night-time level. If the dark period of the eclipse had been longer, recovery from inhibition might have been complete, and it might have become apparent that light-inhibition of flashing rates is not the only cause of the daytime decrease in flashing rate.

Bode, DeSa and Hastings (1963) and Hastings and Keynan (1965), using *G. polyedra* cultures, have shown that normally more luciferin is produced at night than during the day. This was inferred because if night-time flashing was inhibited by temperature or light, more luciferin could be extracted at that time. Under normal conditions, however, night-time flashing apparently utilizes the available luciferin, and more is extracted during the day when flashing is less. It thus appears light inhibition does not affect substrate production, but rather acts upon the stimulus-response mechanism; *i.e.*, it probably decreases the sensitivity of the cells to stimulus. This suggests that flashing of natural populations may be controlled both by the availability of luciferin and by light-inhibition of the sensitivity to stimulus.

The selective advantage conferred upon a dinoflagellate by its ability to luminesce and to control the amount of luminescence is undetermined. McElroy and Seliger (1962) have hypothesized that luminescence first developed to serve a biochemical function during the early evolution of life. As presently found in dinoflagellates, however, the biochemical ability for luminescence is accompanied by at least three mechanisms that serve to control the output of light: (1) a sensitivity to stimulus and an associated effector system, (2) a mechanism whereby sensitivity to stimulus is controlled by light inhibition, and (3) an endogenous rhythm in luciferin production. Energy is required for the production of light, and it seems unlikely that a complex energy-requiring system such as this would evolve and not be lost in such a diverse and widespread group of organisms unless some selective advantage is conferred upon the organisms. More work on the behavior and ecology of luminescence in dinoflagellates is necessary to detect any such advantage.

Although many marine organisms are known to be less luminescent during the day than at night (Harvey, 1952), the only one other than dinoflagellates which has been shown to have an endogenous rhythm is the euphausiid shrimp *Meganycitphanes norvegica* (M. Sars) (Mauchline, 1960). It apparently increases its flashing rate at night even after being kept in the dark for two days. Since the animal has complex photophores with neural and muscular control, presumably luminescence is important in its behavior.

In addition to endogenous and exogenous influences on the luminescence of species of dinoflagellates within a population, luminescence in the natural environment may vary because the species present change and exhibit different characteristics. *G. polyedra* and *Gonyaulax monilata* display similar endogenous rhythms,

whereas *Noctiluca miliaris* gives no indication of an endogenous rhythm (Hastings, 1959). More must be known of the behavior of individuals and cultures of various species before any model can be proposed to describe the behavior of a population composed of many species.

DETERMINATIONS OF LUMINESCENT SPECIES

In order to determine which species of dinoflagellates present during the experiments were capable of luminescence, individual specimens of the species predominant during August and September, 1965, were isolated from the plankton and tested.

Tows were taken on several afternoons, using a nylon net with 35 μ mesh aperture. Water passed through the same net was found to be not luminescent, and it is assumed that all luminescent forms of phytoplankton were captured. Representatives of the dinoflagellates were removed from the sample by micropipette, placed in filtered sea water, and motile individuals were transferred singly from this into 0.5 ml. of filtered sea water in test tubes. The organisms in tubes were kept in the dark until after 2100 hr. before they were tested, so that potential for luminescence would be high when tested, and so that the organisms could recover from the isolation procedure.

The tubes were placed in a light-tight holder in front of the photometer that was used in the previous experiments, and air was bubbled through the water to stimulate the organisms. After testing, the contents were examined to determine if the organisms were still motile, and only those which were motile or which had flashed were considered to have been alive during testing and only these are included in the results. The organisms were placed in a drop of filtered sea water on a slide in a moist petri dish and left overnight. This killed the organisms and often resulted in a loss of protoplasm that simplified drawing and identification.

The organisms tested were drawn with a camera lucida, and were usually placed in glycerine-jelly to facilitate handling and determination of plate structure. Drawings were then compared with more thorough drawings made of specimens of the same species that were not tested, but which were more easily cleared, stained, and manipulated without risk of loss.

Although cell counts of dinoflagellate population density were not made, it was apparent that the populations varied somewhat from day to day. Dinoflagellates were greatly outnumbered by diatoms, but the latter have never been found to be luminescent (Sweeney, 1963). Larger forms which might have been luminescent (such as copepods and ctenophores) had been excluded by filtration. Radiolarians may be luminescent, but were present in very small numbers.

The results of the tests are shown in Table I. Because cells that had been tested were often difficult to recover for identification, only those individuals definitely identified have been included in the table. For example, at least 10 specimens of what was tentatively identified as *Gonyaulax digitale* were tested, and most were luminescent, but were not recovered after testing. Very few of the tested *G. spinifera* flashed, and few were examined for motility after testing. Several specimens of *Gonyaulax* and *Peridinium* believed to be of different species than those identified were examined and were luminescent, but were not identified owing to the lack of specimens. These are listed as *spp.* in Table I.

Of the 12 species and four genera of dinoflagellates present in the Eel Pond plankton during August and September, 1965, 10 species were found to be luminescent. These included the vast majority of dinoflagellate individuals present, and certainly were primarily responsible for the recorded bioluminescence. Of the species found to flash, the following have been previously reported as luminescent: *Peridinium conicum* (Sweeney, 1963), *P. granii* (Ganapati *et al.*, 1959), and *Ceratium fusus* (Lebour, 1925; Sweeney, 1963). Sweeney (1963) tested *P. claudicans* by a similar method and found it not to be luminescent. *Ceratium tripos* has been reported by several authors to be luminescent (Sweeney, 1963), but neither Sweeney nor the present authors could demonstrate a luminescence.

TABLE I
Results of testing individual dinoflagellates for bioluminescence

Species	Number of cells that flashed	Number of motile cells that did not flash
<i>Gonyaulax digitale</i>	2	0
<i>G. spinifera</i> (see text)	2	Several
<i>Gonyaulax</i> spp.	(see text)	
<i>Glenodinium lenticula</i>	0	10
<i>Peridinium claudicans</i>	2	1
<i>P. conicum</i>	2	0
<i>P. granii</i>	4	0
<i>P. leonis</i>	4	0
<i>P. oceanicum</i>	2	0
<i>P. subinermis</i> (Var. <i>punctulatum</i>)	4	0
<i>Peridinium</i> spp.	(see text)	
<i>Ceratium fusus</i>	2	3
<i>C. lineatum</i>	0	10
<i>C. tripos</i>	0	12

The present study has therefore added 6 species to the list of dinoflagellates known to be luminescent.

Negative results in tests such as these must not be considered conclusive, since an organism such as *P. claudicans* or *C. tripos* may sometimes flash and sometimes not. Thus, there appear to be some species always capable of luminescence, some that never luminesce, and others which are capable of luminescence only under certain conditions.

TAXONOMY

No thorough taxonomic work has been done on the armored dinoflagellates of the region directly south of Cape Cod, and identification must be made with reference to Lebour (1925) and Schiller (1937), who deal primarily with European and oceanic forms. The species referred to as *Glenodinium lenticula* (Bergh) Schiller, and several species of *Peridinium* are in need of revision. It is deemed desirable to illustrate and note the characteristics of the five species given below so that our identification will be meaningful in case of future revision. The other species tested seem secure in their taxonomic position,

Glenodinium lenticula (Bergh) Schiller (Fig. 4, g-j).

This species is very variable and has a lengthy synonymy (Schiller, 1937). The form worked with here varies considerably within the population. It may have four apical plates; *i.e.*, a plate that some authors have described as the second intercalary (Schiller, 1937, p. 104; Figs. 95, 96) actually reaches the apical pore. Individual specimens may or may not have a small asymmetrical intercalary plate between precingulars 2'' and 3'' and apicals 2' and 3'. Schiller (1937) illustrates forms with and without this plate. If this plate is not present, there are 7 precingulars, the third reaching further toward the apex in place of the asymmetrical intercalary; if the intercalary is present, only 6 precingulars are found. The widths of the pre- and postcingular plates are very variable, and in some cases these plates are barely visible. The theca is punctate, the sutures are often broad and striated, the lists have very fine supporting spines, and the apical pore may or may not be strongly developed. Plate structure of the species described here: 4 apicals, 0 to 1 intercalary, 7 or 6 precingulars, 5 postcingulars, and 2 antapicals.

Peridinium conicum (Gran) Ostenfeld and Schmidt (Fig. 4, d-f).

In the past this species has been confused with both *P. pentagonum* Gran and *P. leonis* Pavillard, and the differences between them are slight. Lebour (1925, p. 111) and Schiller (1937, p. 237) separate *P. pentagonum* from *P. conicum* on the basis that the former has solid antapical spines, and that its right half is larger than its left, but this is not shown clearly in their figures. These characters are nevertheless sufficient to identify the species discussed here.

Peridinium leonis Pavillard (Fig. 4, k-n)

This species is easily separated in our samples from the previous one although earlier descriptions (Schiller, 1937, p. 236) indicate a wide variation. It may be distinguished from other species described here by the following characters: cell dorso-ventrally flattened, broad lists with prominent spines, girdle forms an acute angle with cell axis, surface with reticulations appearing striated on some plates, first apical plate narrower than in *P. conicum*. Schiller (1937, p. 236) and Lebour (1925, p. 112) have separated it from *P. conicum* on the basis of its much more prominent lateral sutures, but this is not always evident in the individuals investigated here. The species here corresponds most closely to those described by Dangeard (1927) and Klement (1964), and probably several similar species are included in *P. leonis* in the summary by Schiller (1937).

Peridinium granii Ostenfeld (Fig. 4, a-c).

This species is easily confused with *P. brochii*. The only character separating them is the asymmetry of the dorsal plate structure, and this is variable (Schiller, 1937, p. 189). It is easily separated from the other species investigated here, however, by the structure of the first apical plate. The present form corresponds most closely to that illustrated in Lebour (1925, p. 124). It is characterized by the structure of the first apical plate and the asymmetry of the dorsal plates.

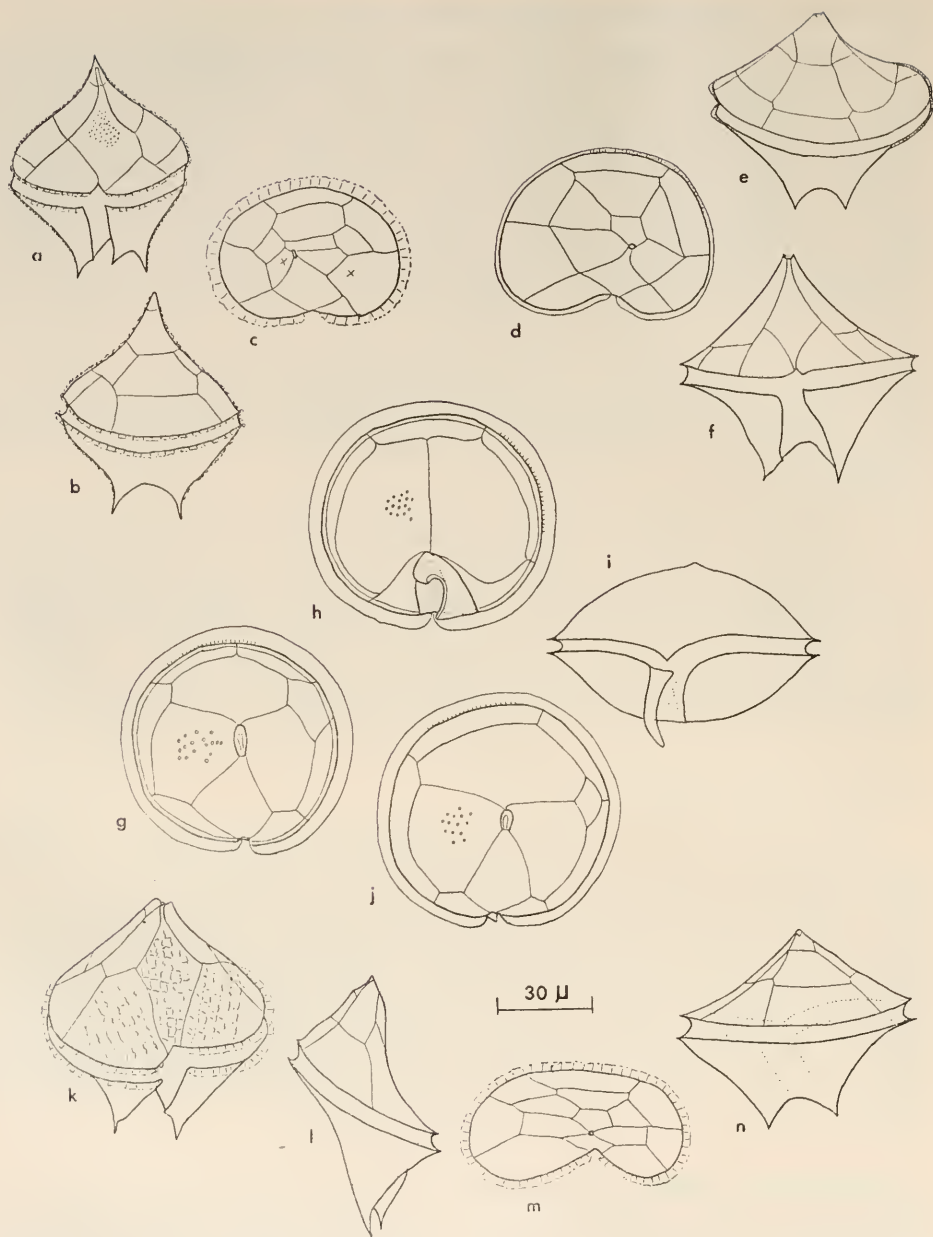


FIGURE 4. Dinoflagellates whose taxonomy is discussed in text. a-c, *Peridinium granii*; d-f, *Peridinium conicum*; g-j, *Glenodinium lenticula*; k-n, *Peridinium leonis*.

CONCLUSIONS

Most dinoflagellate species and individuals taken from Eel Pond during this study were luminescent and these were sufficient in abundance to explain all the luminescence recorded. This is probably the case in many marine environments. Macroscopic organisms capable of luminescence were removed by filtration, and the only microplankton constituents capable of luminescence and present in sufficient numbers were dinoflagellates.

Dinoflagellate luminescence is commonly a cause of light production in surface regions of the ocean (Harvey, 1952; Hastings, 1963) and more knowledge is needed of the luminescent behavior of individuals and cultures of the various species. The effects of temperature, depth and other environmental conditions are unknown. Spontaneous luminescence without stimulation was observed in the laboratory, but is very variable and its extent in the natural environment is not known. Much work is needed on the ecology of dinoflagellate luminescence.

The rate of luminescent flashing of natural populations following stimulation is greatest at night, is controlled by an endogenous diurnal rhythm, and is inhibited by light. The sensitivity to light-inhibition is also controlled by an endogenous rhythm, and is greatest during midday when flashing is least. Thus in the natural environment, light-inhibition and an endogenous rhythm act together in decreasing stimulated daytime luminescence.

The authors would like to thank Dr. G. L. Clarke of Harvard University and Woods Hole Oceanographic Institution for his continued guidance and encouragement and for the loan of equipment and facilities, Dr. Frank Round of the University of Bristol, England, for his encouragement and assistance in the taxonomic work, and Drs. R. Backus and C. S. Yentsch and various other members of Woods Hole Oceanographic Institution who have made valuable suggestions and criticisms throughout the work.

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FEEDING BEHAVIOR AND REPRODUCTIVE CYCLES IN *PISASTER OCHRACEUS*¹

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A number of studies have shown the importance of the relation between energy intake and reproductive effort in higher organisms. Lack (1954) summarizes a mass of data indicating, especially for birds, the dominant role played by the availability of food in determining reproductive strategy. His major conclusion, based on different degrees of reproductive success and survival, implicates a complex interaction with the environment. Among invertebrates the influence of natural selection on reproductive patterns and processes is poorly understood. In the field, the dependence of gamete production on food has been shown for copepods (Marshall and Orr, 1955), rotifers (Edmondson, 1965) and for a few other organisms. Laboratory work is more convincing but generally less applicable. Experimental studies on *Daphnia* (Richman, 1958), a rotifer (King, 1965), as well as any study showing a relationship between rate of population growth and food level, can be thought of as giving evidence concerning the general dependence of the reproductive performance on the nutritional state of the population.

Among marine macro-invertebrates, a number of studies have suggested such dependence, but ecological data on feeding have been lacking. For example, Farmanfarmaian, Giese, Boolootian and Bennett (1958) have indicated an inverse relationship between the size of the gonads and pyloric caeca of two carnivorous sea-stars, *Pisaster ochraceus* and *P. brevispinus*. Pearse (1965) suggested that different populations of a probably omnivorous Antarctic sea-star, *Odontaster validus*, varied in reproductive activity according to local differences in primary production. Boolootian, Farmanfarmaian and Giese (1962) have demonstrated reciprocal relationships between genital and hepatic tissue in the abalones *Haliotis cracherodii* and *H. rufescens*, as have Lawrence, Lawrence and Giese (1965) for the algivorous chiton, *Katharina tunicata*. Most of these authors have suggested that the digestive glands are used to stockpile nutrients during the months when feeding is most efficient. Later these storage products are transferred to the maturing gonads. The hypothesis that changes in hepatic tissues are correlated with feeding can be tested most feasibly in a carnivorous species. Qualitative and quantitative aspects of the nutrition of carnivores are usually easier to follow under natural conditions since direct observation of ingested prey is possible.

The starfish, *Pisaster ochraceus* (hereinafter referred to as *Pisaster* unless another species is indicated), was chosen for this study for several reasons. *Pisaster* is usually abundant in the rocky intertidal region of San Juan Island,

¹ This paper is a condensed version of a thesis submitted to The University of Washington in partial fulfillment of the requirements for the degree of Master of Science.

Washington, as well as on most rocky shores on the West Coast of North America. This, coupled with its relatively limited mobility and large size, permits large numbers to be obtained from repetitive sampling of local, discrete populations. Feeding is easily observed. As reported by Feder (1956, 1959), *Pisaster* uses its tube feet to force open prey, or to wrench it off the substrate; the cardiac stomach is then everted onto the exposed soft tissues. Consequently, by turning these starfish over, the incidence of feeding and the identity of the prey can be observed. *Pisaster* occupies the position of "top predator" (Paine, 1963) in its community, preying on the members of several lower trophic levels, but with no important predators of its own (Paine, 1966). As noted above, Farmanfarmaian *et al.* (1958) have demonstrated inverse gonad-pyloric caeca cycles. Mauzey (1963) has previously noted that there is also a seasonal feeding cycle. The present paper reports the nature of the interrelationships of the organ and feeding cycles and discusses the ecological consequences and implications.

THE STUDY AREA

The study was carried out at Lonesome Cove, situated at the northeast tip of San Juan Island (Latitude, 48° 37'20" N, Longitude, 123° 6'30" W). The area is scoured by a strong tidal current. Wave action is minimal; except for winter storms there is no more than would be expected on a medium-sized lake. The tidal range is from minus 3.5 feet to plus 9.0 feet. The temperature is at its maximum of about 13° C. in July; the minimum of about 6° C. is reached in January. Salinity is relatively constant, varying from about 29‰ to 31‰.

The intertidal flora and fauna correspond generally to those described from Vancouver Island by Stephenson and Stephenson (1961; especially Brandon Island), although the zonation is not as distinct. There is a splash zone above plus 7.0 feet dominated by *Littorina sitkana* and *L. scutulata*. Below this, *Littorina* is less numerous and interspersed among other organisms. There is a *Balanus-Fucus* zone from about 3 to plus 7 feet and a bare zone, with a few scattered barnacles, below this to the 0 tide level. Below 0, to an indefinite boundary several feet below extreme lower low water, there is an almost continuous covering of brightly colored crustose coralline algae (mostly *Lithothamnion* spp.) and a gradual increase in the number and variety of brown algae, *Laminaria* spp., *Alaria valida* and *Nereocystis luetkeana*. There is a summer covering of several green algae similar to *Ulva*, extending to plus 2 or 3 feet. All the algae but *Fucus* are markedly seasonal; the low intertidal and subtidal regions are almost bare in the winter, while the rocks are usually completely covered in the summer months. There are very large populations of urchins (*Strongylocentrotus dröbachiensis* and *S. franciscanus*) that undoubtedly account for the algal disappearance following the summer's prolific growing season. These urchins are generally covered at low tide, but move up to plus 4 feet to feed during nocturnal high tides.

The limpet, *Acmaea persona*, is prevalent in the upper part of the *Balanus-Fucus* zone. *A. digitalis*, *A. pelta* and *A. scutum* are abundant below the usual range of *A. persona* down to the beginning of the coralline algae zone. *A. mitra* occupies this latter zone, but is never very common. Several predatory snails, *Thais lamellosa*, *Scarlesia dira* and occasionally individuals of *T. emarginata* and *T.*

canaliculata, occupy the *Balanus-Fucus* zone. A set of *Mytilus edulis* occurs most springs in this zone, but few survive the summer. The chiton, *Katharina tunicata*, is prevalent from the lower part of the *Balanus-Fucus* zone well into the coralline algae zone. Other chitons, including *Tonicella lineata* and several species of *Mopalia*, share the same range, but are much less abundant. There are many hermit crabs, *Pagurus* spp., and shore crabs, *Hemigrapsus nudus* and *H. oregonensis*.

FEEDING OBSERVATIONS

Pisaster is one of the most conspicuous animals on wave- or current-swept rocky shores from Sitka, Alaska, to Ensenada, Mexico (Ricketts and Calvin, 1952). Most of the population is confined to the intertidal zone. I have only occasionally observed animals as deep as 30 feet below mean lower low water (the zero of West Coast Tide Tables). Feder (1956) indicates that this is the maximum depth for *Pisaster* in Central and Southern California.

Observations on feeding were made by skin-diving over the intertidal zone during high tide. This phase of the tidal cycle was chosen because the animals are then at their peak of foraging activity (Mauzey, unpublished data). Each individual was removed from the substrate and its oral surface examined. An everted stomach was taken as evidence of feeding; usually the prey could be seen in the folds of the stomach. These observations, together with a size estimate of the predator, were recorded on a plastic card. During the first part of the study, May, 1962, through July, 1963, sampling dives were made twice monthly. During the remainder of the study the sampling interval was lengthened to once a month because analysis of the initial feeding data indicated that all trends discussed below would be apparent with this longer sampling interval. From May, 1962, through April, 1963, each dive was terminated after 100 animals had been observed. After April, 1963, the entire sampling area was searched during each dive, and all specimens of *Pisaster* present were counted. This change was made because a seasonal change in abundance was noticed. These samples indicate that the population varies from about 100 in the winter to about 200 in the summer. There are indications that this is due in part to more starfish being hidden in crevices and under rocks in the winter, and in part to a seasonal movement into deeper water.

The feeding results for the entire period (22 months) are given in Figure 1 which is based on observations of 3,820 individuals, of which 1,364 individuals (35.4%) were feeding. Since one sea-star sometimes feeds on more than one prey species at a time, there are 1,557 observations of feeding on particular prey species. The category, *Balanus* spp. includes predominantly *B. cariosus*, but a few small *B. glandula* were also taken. The category *Acmaca* spp. in the feeding observations includes predominantly *A. pelta* and *A. scutum*, but *Pisaster* also eats a few *A. digitalis* and *A. persona* that are in its range. Of all observations 81% are on *Balanus* spp., *Acmaca* spp. and *Mytilus edulis*; the rest each account for 5% or less.

In an additional 30 observations the stomach was everted but no prey could be found. These are not included in Figure 1. The most likely explanation is that these represent animals engaged in flagellary-mucous feeding on detritus (Anderson, 1960; Mauzey, 1963; Pearse, 1965). An alternative explanation, that prey was present but not observed, is less likely since these everted stomachs were carefully searched, often to the extent of damaging the thin-walled organ.

These observations are very similar to those of Feder (1959) in California. He reports somewhat greater feeding on barnacles (*Balanus glandula*, *B. nubilis* and *Tetraclita squamosa rubescens*) (57.0%) and on *Mytilus californianus* (17.0%) but less feeding on *Acmaea* spp. (4.8%) and on chitons (mostly *Mopalia muscosa*) (4.5%). There is a large difference with respect to *Katharina tunicata*, the chiton mainly eaten in the San Juan Island area. Only 0.2% of his observations were on this species, as compared with 3.8% of mine. In both studies, a few organisms are fed on heavily, while a large number is eaten only occasionally.

Although *Balanus*, *Acmaea* and *M. edulis* are the numerically dominant prey, they represent a much smaller percentage of the total biomass of food. Dry weight *versus* length correlations were established for the six most prominent species

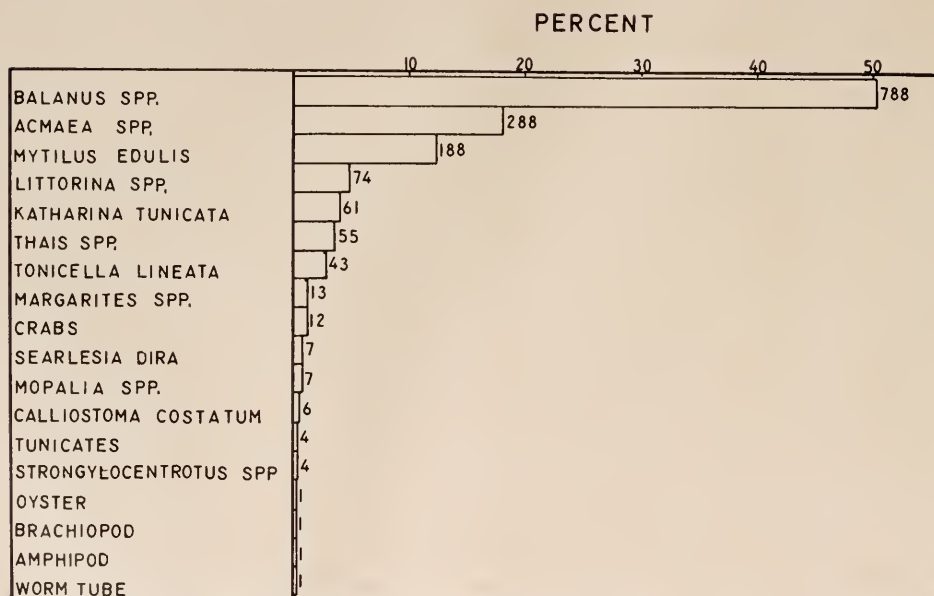


FIGURE 1. Percentages of *Pisaster* feeding on indicated prey, summed over the entire study period, March, 1962, through January, 1964. The number observed feeding on each prey category is given at the end of the bar.

consumed by *Pisaster* (Mauzey, unpublished data). Only those parts of the prey that are actually eaten by *Pisaster* were weighed, *i.e.*, shells, and the girdle of chitons, were omitted. The size and number of all prey could not be recorded in the field due to lack of time underwater. Therefore, I estimated the average size and number of each prey for all feedings, based on impressions gathered over the entire period of the study. This method permits only a rough estimate of the biomass ingested (Table I). Research in progress suggests that the seasonal pattern reported here is typical for *Pisaster* at Lonesome Cove. On the basis of dry weight, chitons are the most important prey; *Balanus* and *Mytilus edulis*, because of their small size, are of secondary importance. The importance of *Acmaea* and *Littorina* is somewhat reduced, and that of *Thais* spp., a carnivorous

whelk, is greatly increased, but it still remains a small part of *Pisaster's* diet. Paine (1966) has found approximately the same reversals in importance on the outer coast of Washington.

When these data are observed with respect to time, a definite feeding cycle is apparent in terms of per cent feeding, number of individuals eaten, and dry weight ingested. The percentages of *Pisaster* that were observed feeding in each sample, and the estimated dry weight ingested are plotted in Figure 2a. The calculations for

TABLE I

Dry weight data for the six most important of Pisaster's prey. Since the data are calculated on the basis of a common number observed in each sample (100), the numbers in the total column do not agree with Figure 2, which is based on the uncorrected data. The data from the single winter are doubled to allow comparison with the data from two summers

	Prey organism					
	<i>Balanus</i> spp. (<i>B. cariosus</i> , <i>B. glandula</i>)	<i>Acmaea</i> spp. (<i>A. pelta</i> , <i>A. scutum</i>)	<i>Mytilus</i> <i>edulis</i>	Chitons (<i>Katharina</i> , <i>Tonicella</i> , <i>Mopalia</i> spp.)	<i>Littorina</i> spp. (<i>L. sitkana</i> , <i>L. scutulata</i>)	<i>Thais</i> spp. (<i>T. lamellosa</i> , <i>T. emarginata</i> , <i>T. canaliculata</i>)
Average size ingested (cm.)	1.60	2.10	1.00	7.50	0.70	6.00
Dry weight of ingested size (gm.)	0.04	0.10	0.01	2.50	0.03	0.60
Average number per feeding	5	3	5	1	3	1
Dry weight per feeding (gm.)	0.20	0.30	0.05	2.50	0.10	0.60
Number ingested						
Winter (Dec. '62–Mar. '63)	20	18	10	48	2	4
Summer (June–Sept. '62 & '63)	479	177	90	30	48	14
One year (May '62–May '63)	367	213	100	77	48	27
Total (Mar. '62–Jan. '64)	620	249	140	106	66	39
No rank						
Winter	2	3	4	1	6	5
Summer	1	2	3	5	4	6
One year	1	2	3	4	5	6
Total	1	2	3	4	5	6
Dry weight ingested						
Winter	4.00	5.40	0.50	120.00	0.20	2.40
Summer	95.80	53.10	4.50	75.00	4.80	8.40
One year	73.40	63.90	5.00	192.50	4.80	16.20
Total	124.00	74.70	7.00	265.00	6.60	23.40
Weight rank						
Winter	3	2	5	1	6	4
Summer	1	3	6	2	5	4
One year	2	3	5	1	6	4
Total	2	3	5	1	6	4

the dry-weight curve were made with the data of Table I. This introduces some error since the average size of the shorter-lived species changes during the year, *e.g.*, the barnacles are smaller in the spring just after settling than in the fall after a summer of growth. These small barnacles provide a large proportion of the summer prey, but some that are several years old are also eaten. In order to compare the biomass of prey ingested on each dive, a correction must be made for the different number of starfish observed on different dives. In Figures 2a and b, observations are corrected to 100 animals per dive; in Table I, to 200 per month, or two dives of 100 animals each. Figure 2b shows the number of starfish feeding on various items; starfish feeding on two prey species are recorded twice. Figure 2c plots the per cent of *Pisaster* feeding on a particular prey as a percentage of those feeding.

Chitons play an unexpectedly important role in *Pisaster*'s nutrition. Few are ingested at any time, and, except for a slight drop in the summer, the rate at which they are eaten appears to be constant (Fig. 2b). In the winter chitons constitute almost the sole food, while most of the *Pisaster* individuals are feeding on other prey in the summer. The comparatively large size of chitons among *Pisaster*'s prey, and a pronounced seasonal behavior pattern of the predator, account for these observations. In the summer, the sea-stars are scattered singly over the intertidal from about plus 5 feet to minus 2 feet tide level, while in winter from 5 to 25 starfish may clump together in crevices and other protected areas between about zero and minus 4 feet tide level. The same crevices are occupied from dive to dive, apparently by the same starfish, since individuals which had distinctive color patterns were observed in the same crevices for several dives. These clumped animals, characterized by a low incidence of feeding, eat primarily chitons. These prey, the only ones that occur commonly below the zero tide mark, form the major part of *Pisaster*'s winter diet, apparently because their grazing movements bring them into the predator's winter clumps.

Preliminary observations suggest that *Pisaster* prefers *Mytilus*, and, in fact, may have evolved in relation to the dense populations of *M. californianus* on the exposed coast. At Lonesome Cove, *Pisaster* seems to devour preferentially individuals of the species *M. edulis* within a relatively short period after their settlement. In areas like this, *Pisaster* must feed on such large potential prey as chitons in order to sustain themselves, although laboratory observations suggest that these are eaten only with great reluctance in the laboratory.

SEASONAL CHANGES IN GONAD AND HEPATIC TISSUES

Giese (1959) describes a method for assessing the reproductive cycle of an animal by periodically determining a gonad index, a measure of the ratio of gonad size to body size. This method assumes that in individuals large enough to be mature, a spent or immature gonad is small, and a ripe gonad is large. A plot of successive determinations on samples of a population indicates the average reproductive state of the population with respect to time. Spawning is indicated by a sharp drop in the gonad index. A similar method can be applied to indicate the condition of food-storage organs.

Farmanfarmaian *et al.* (1958, p. 356) have published their procedures for determining such gonad and hepatic indices for *Pisaster* from the Central California

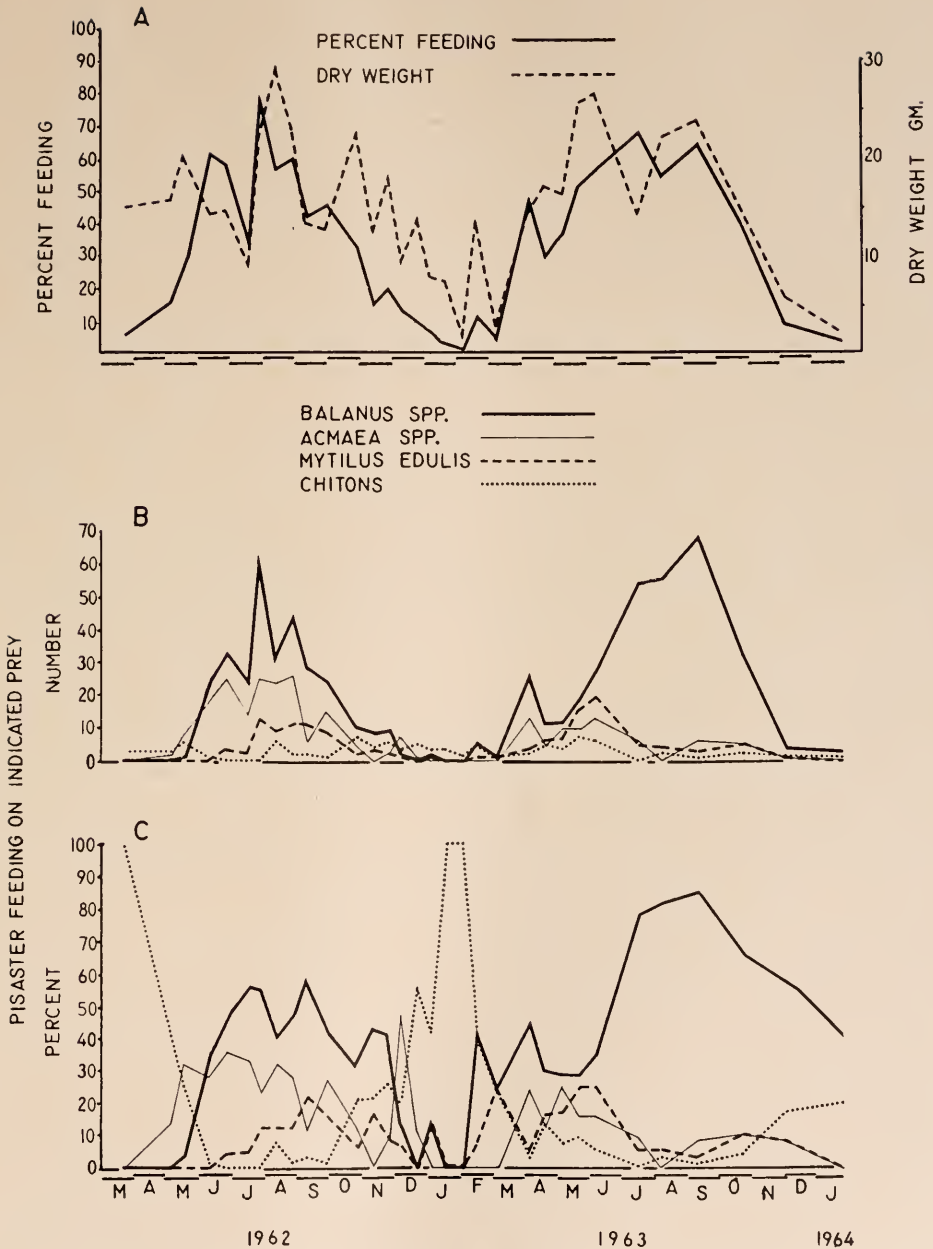


FIGURE 2. The seasonal feeding pattern of *Pisaster*. A. The per cent of the animals observed feeding, and an estimate of the dry weight ingested at each sample. B. The number of *Pisaster* observed feeding on the four most commonly eaten prey. These data are corrected to a common basis of 100 animals observed in each sample. C. The data of B plotted as a per cent of those feeding, showing that chitons are the only prey at certain times of the year. The legend above B applies to both B and C.

coast: "Ten specimens were gathered monthly. Each specimen was weighed after blotting and slit aborally along the arms towards the center of the animal with a pair of scissors. The gonads were removed and the volume was determined by its displacement of a known volume of water in a graduate cylinder. The ratio of gonad volume to body weight $\times 100$ was taken as the gonad index. The digestive gland was weighed and the ratio of digestive gland to body weight $\times 100$ was taken as the hepatic index." Measurements of the specific gravity of the gonads and hepatic caeca indicated that the specific gravity does not deviate significantly from that of water, obviating the necessity of making conversion to weight. The results from this three-year study by Farmanfarmaian *et al.* show a peak from about March through May, and a rapid drop in May or June, associated with gamete release. The gonad index increases again in October or November, and gradually climbs to its spring peak. In one year the peak was lower, and the decline earlier and more gradual.

The hepatic index *generally* shows an inverse relationship to the gonad index. Farmanfarmaian *et al.* (*loc. cit.*) suggest that this is due to the transfer of stored glycogen, lipid and protein (shown to be present in the pyloric caeca by Greenfield, Giese, Farmanfarmaian and Boolootian, 1958) to the developing gonad. They further state that this does not seem to be correlated with a seasonal feeding cycle. However, starved individuals did show shrunken gonads as well as shrunken pyloric caeca (*cf.* also Feder, 1956), indicating that if seasonal differences in the population's feeding pattern do exist, they are likely to have an important effect on the size of these organs.

The method described above was followed closely in the present study, except that the volume, rather than the weight, of the pyloric caeca was used to calculate the hepatic index. The substitution should not affect comparisons, due to the closeness of the organ's specific gravity to 1.0. The whole animals were drained on paper toweling for about 15 minutes before weighing; the excised organs were also blotted before measurement, for about 5 minutes. The hepatic index (Fig. 3) rose during the autumn in 1962 and 1963, reached a plateau in March, 1963, and dropped rapidly in June. As in the earlier study by Farmanfarmaian *et al.*, the female gonad index was somewhat higher than that of the males.

Spawning was observed in laboratory tanks in early May and again on June 7, 13, and 14, 1963. No spawning was observed in the field that summer; in other summers I have observed *Pisaster* spawning in June, July and August. Moreover, *Pisaster* gametes are often difficult to obtain for embryology classes during the latter half of the summer, whereas they are usually readily available in the first half of the summer. The release of most of the gametes would seem to have taken place in the middle of June, 1963, based on the gonad index and scattered laboratory observations (as was also the case in 1965); smaller numbers are released throughout the rest of the summer, accounting for the continued slight fall of the gonad index. In California spawning appears to be more abrupt, and to occur somewhat earlier. According to Giese (1959) *Pisaster* in California spawns from April to May; only occasionally does the spawning extend into June. The gonad index there is uniformly low throughout the summer months.

As was true in California, the hepatic index for Lonesome Cove animals is approximately inverse to the gonad index. An hepatic index minimum is reached

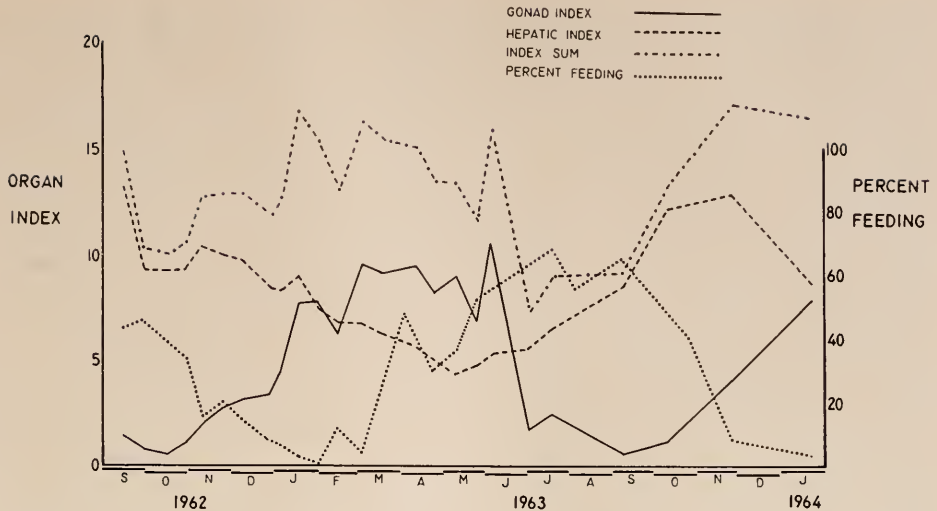


FIGURE 3. Seasonal changes in the gonad and hepatic indices, per cent feeding, and the sum of the gonad and hepatic indices. Note that the organ indices are approximately inverse to each other, and that the feeding curve is similar to that of the hepatic index, but delayed about 4 months.

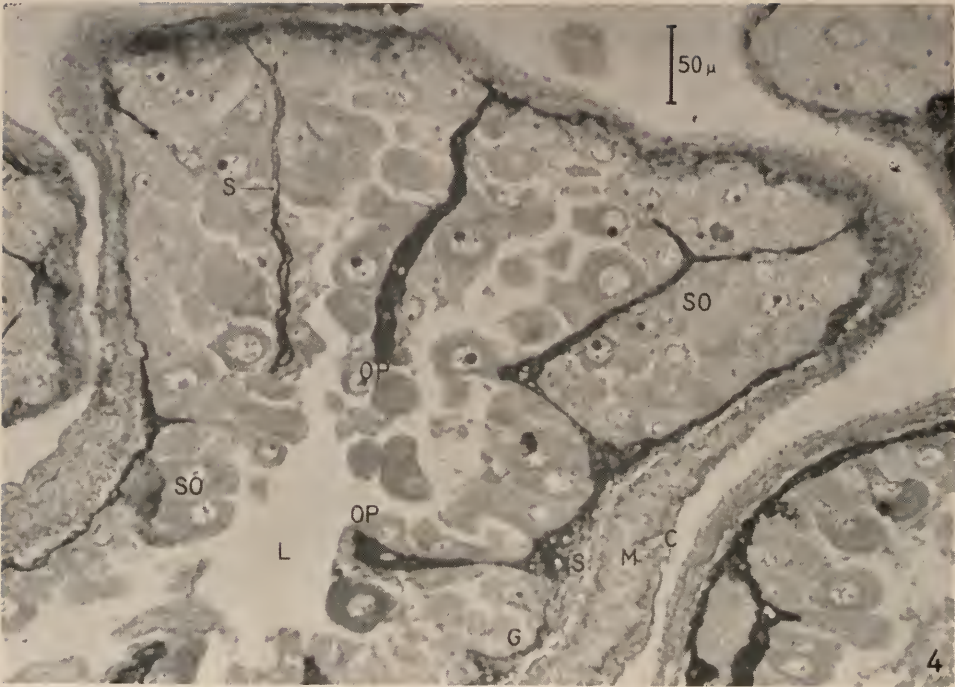
in May; the index rises all summer to a peak in November, and falls until the next spring (Fig. 3). There is no significant difference between the male and female hepatic index.

HISTOLOGY

Specimens of the excised organs of each starfish obtained at each sampling were preserved in Bouin's fluid for subsequent histological observations. These provide both general confirmation of the gross changes in size, and some details as to what is involved in these changes. The tissues were imbedded in paraffin, sectioned at $5\ \mu$, and stained with Harris' hematoxylin and eosin, Mallory's triple stain, or the periodic acid-Schiff routine (PAS), counterstained with Harris' hematoxylin (Pantin, 1946; McManus and Mawry, 1960). Salivary amylase digestion was used in conjunction with some of the PAS material.

Sections were made of the ovaries of three females from samples collected on September 14, 1962 (Fig. 4), December 25, 1962, and April 8, 1963 (Fig. 5). The gonad indices of the females were 0.73, 3.59 and 22.91, respectively; the gonad indices of the samples from which they were taken were 1.43, 3.38 and 9.47, respectively.

In September, the oocytes (Fig. 4) have diameters between 10 and $50\ \mu$; in December, they range from about $10\ \mu$ to $150\ \mu$; in April (Fig. 5) there are two size groups, one about $150\ \mu$, and the others less than $20\ \mu$. Below about $20\ \mu$, they are PAS-negative; above that size the oocytes become progressively more and more PAS-positive. The very intensely PAS-positive oocytes of $150\ \mu$ are probably "mature," although they can only be fertilized if released through the oviducts. Living eggs thus obtained measure about $200\ \mu$; the $50\ \mu$ difference is



FIGURES 4-5.

probably a fixation artifact. The small, PAS-negative oocytes present in April are probably those that will be spawned one year hence; *i.e.*, complete oogenesis may take more than one year. However, since some "mature"-sized oocytes are present in September, December and April, there could be continual, or almost continual, production of new oocytes, with maturation taking perhaps eight months (April or September to December or May).

In the ovaries collected in September and December, invaginations of the basement membranes of the germinal epithelium produce ovarian folds (Fig. 4), which have not been previously described from asteroid ovaries. The developing oocytes occur along the germinal epithelium both on and between the folds. Between the basement membrane on either side of the folds, and between the basement membrane and the rest of the ovarian wall there is a sinus. This sinus has been reported before by several investigators, including Chia (1964) in *Leptasterias*, and Delavault (1961) in *Echinaster sepositus*. Both the basement membrane and the coagulated contents of the sinus are strongly PAS-positive (see Fig. 4). In the mature ovary, the presence of many mature ova seems to stretch the rest of the ovarian wall, and flatten out the folds. The sinus is reduced and no longer PAS-positive; the basement membrane is still PAS-positive, but appears much reduced, perhaps due to being stretched (see Fig. 5). Chia (1964) postulates that the sinus serves to supply nutrients to the developing oocytes. This hypothesis is supported by my observations that (a) only mature gametes are free in the lumen, (b) this space becomes reduced as a larger proportion of the ova become mature, and (c) the contents are PAS-positive when the rate of transfer of nutrients to the gonads is heaviest.

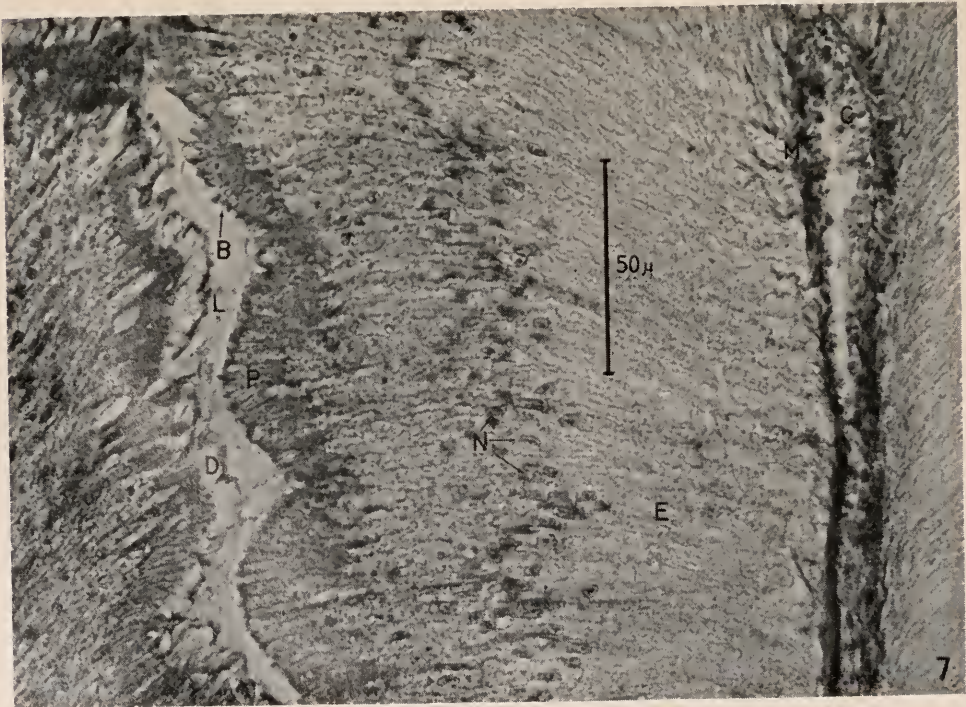
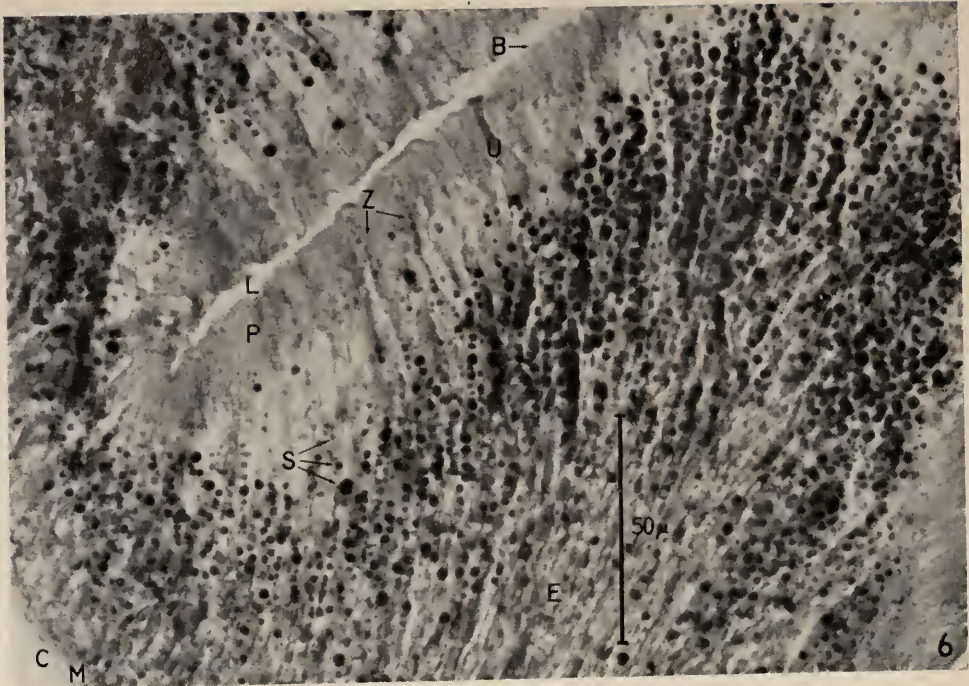
Spermatogenesis has not been followed, but some observations seem to be in general agreement with the descriptions of Cognetti and Delavault (1960) and Pearse (1965). Motile sperm are present in smears of testes from at least a few specimens of *Pisaster* at all times of the year. Testes, as well as ovaries, collected in the spring tend spontaneously to release very large numbers of gametes; it is necessary to collect these shed gametes carefully to get an accurate gonad volume measurement. A preliminary section of the testes indicates the presence of the spermatic papillae described by the above authors; the interior of these is occupied by a fold of basement membrane enclosing a sinus whose contents are PAS-positive, as in the ovary. This does not appear to be the case in *Odontaster* (Pearse, 1965) or *Echinaster* (Cognetti and Delavault, 1960).

Salivary amylase treatment of sections of ovaries collected in December indicates no detectable change in the PAS-positive reaction of either the gametes themselves, or the coagulated material within the sinuses. Similar results have been obtained by Chia (1964). This indicates that the material is not glycogen, agreeing with

FIGURE 4. Photomicrograph of a *Pisaster* ovary collected in September, 1962. The tissue was fixed in Bouin's fluid, imbedded in paraffin, sectioned at 5 μ , and stained with PAS and hematoxylin. The structure of the ovarian wall, the ovarian folds, the sinus and the preponderance of small oocytes are visible.

Figure 5. Photomicrograph of a *Pisaster* ovary collected in April, 1963. Same technique as in Figure 4. The many large oocytes and the few very small ones should be noted.

Key to abbreviations used in Figures 4 and 5: C, coelomic epithelium; G, germinal epithelium; L, lumen; LO, large oocytes; M, middle layer of ovarian wall; OP, distal end of ovarian fold; S, sinus; SO, small oocytes.



FIGURES 6-7.

the observation of Greenfield *et al.* (1958) that glycogen is a minor constituent of the gonads of *Pisaster*, averaging only about 0.35% of the dry weight.

Sections of the pyloric caeca generally substantiate the histology of these organs reported by Anderson (1953) for *Asterias forbesi*, and by Chia (1964) for *Leptasterias hexactis*. There is first an outer peritoneal layer, then a layer containing connective, nervous, and muscular tissue elements, and, finally, the epithelial layer of very tall, slender columnar cells. The latter is mostly responsible for the thickness of the caecal wall. There are four kinds of cells in this epithelium: (1) special current-producers, (2) zymogen or secretory cells, (3) storage cells, and (4) mucous cells. Since the pyloric caeca of *Pisaster* are larger and more folded than those of the other starfish studied, the special current-producers that occur mainly on the oral and aboral aspects of the central duct have not been seen in my sections through the lobes of the pyloric caeca. The storage cells comprise the bulk of the remainder of the epithelial layer, with many scattered zymogen cells, and relatively few mucous cells (see Figs. 6 and 7).

The location, staining qualities and seasonal appearance of the granules abundantly present in Figure 6 suggest that they represent nutrients stored in the pyloric caeca during the summer feeding period. These storage granules seem to be within the storage cells identified by Anderson (1953) and Chia (1964). When stained with Mallory's triple stain, these granules can be distinguished as two types which form overlapping bands across the long axis of the storage cells. The distal band stains yellow; the proximal, wider band, blue. The proximal band is PAS-positive. In pyloric caeca from *Pisaster* of low hepatic index (Fig. 7) there are very few, or no storage granules present. There is a band of nuclei in the middle portion of the cells, presumably obscured by storage granules in Figure 6. The major morphological differences between Figures 6 and 7 thus relate easily to seasonal patterns of transfer and storage of energy-rich materials in the hepatic caeca.

DISCUSSION

The information in the preceding sections on feeding, patterns of organ morphology, and reproductive cycles permits clarification of the relationship between these phenomena and a discussion of the evolution of *Pisaster's* seasonal behavior.

The organ indices and the per cent feeding for each sampling are plotted together for comparison in Figure 3. These curves indicate a functional relationship between the processes they measure. Food ingested during the summer is stored in the pyloric caeca; investigations by Anderson (1953) and Ferguson

FIGURE 6. Photomicrograph of a *Pisaster* pyloric caecum collected in September, 1962. The tissue was fixed in Bouin's, imbedded in paraffin, sectioned at 5 μ , and stained with Mallory's triple stain. Note the presence of zymogen cells, mucous cells, and storage cells filled with many storage granules. In this and the following figure, the epithelial layer (E) occupies almost the entire figure.

FIGURE 7. Photomicrograph of a *Pisaster* pyloric caecum collected in April, 1963. Same technique as in Figure 6. Note the absence of zymogen granules and storage granules.

Key to the abbreviations used in Figures 6 and 7: B, brush border; C, outer epithelial layer; D, distal disintegration; E, epithelial layer; L, lumen; M, middle layer of the wall of the pyloric caecum; N, nuclei of cells of the epithelial layer; P, pigment layer; S, storage granules in storage cells; U, mucous cells; Z, zymogen granules in zymogen cells.

(1964a) strongly suggest that these are the major storage organs of asteroids. There is a 2-4 month interval between the response maximum and minima of the dry weight ingested (Fig. 2a) and the hepatic index curves (Fig. 3). This lag is explained by the fact that the size of the pyloric caecum represents a temporal summation of the excess of nutrient over metabolic use. Reduced respiratory costs associated with declining water temperatures and lessening movement in the fall must more than offset the reduced caloric income, and the hepatic index continues to rise even after the maximum value of the dry weight ingested. In the spring the pyloric caeca continue to decrease until feeding supplies more nutrient than is used.

The gonads begin to grow in size in the fall, employing material from the pyloric caeca accumulated from summer feeding. Ferguson (1964a, 1964b) gives experimental verification of nutrient transfer from the digestive glands to the other tissues of starfish through the coelomic fluid, even though the concentration of nutrient in this fluid is very low at any one time. As the gonads increase in size, the removal of stored nutrient causes a decline in the size of the pyloric caeca. Finally, the gonad index drops dramatically when the animals spawn in June.

Pisaster probably spawns in the late spring, with the effect that the larvae are in the plankton during the summer when the larval food supply is at its greatest abundance. The length of time spent in the plankton can be inferred from a study by Quayle (1954) at Nanaimo, B. C. He observed young starfish on strings of oyster shells set out in conjunction with a study of oyster settling. These had been exposed for oyster settlement in August, 1952, at which time the starfish must also have settled (Quayle, 1954). One can reasonably postulate a June spawning time for these starfish for two reasons: (1) the two known spawning periods for *Pisaster* are very close: May, generally, for central California (Farmanfarmaian *et al.*, 1958) and June, for the San Juan Island region, and (2) the use of the latter period is justified by the proximity (50 miles) of Nanaimo to my study area. Further, since Quayle reports a "heavy settlement" of the young sea-stars, this settlement probably resulted from the peak spawning period in June. Therefore the larval period must last about two months, from June to August, when the plankton is richest, the factor which must ultimately set the timing of *Pisaster*'s reproductive cycle.

The fundamental reason why, in *Pisaster*, the volume of pyloric caeca varies seasonally must be related to a pronounced advantage of feeding during the summer. If not, *Pisaster* could feed and elaborate gametes continuously. To follow the former strategy, some means of energy storage is essential, this requisite being met by the pyloric caecum. Unless the pyloric caeca can serve this function, feeding must be greatest at the time of gonad growth. The loss of approximately 10% of the body weight in gametes (up to 23% in some individuals) must consume a considerable proportion of the energy assimilated yearly. On an ash-free dry-weight basis, these percentages are even higher since calcareous structural material forms a considerable part of the body wall. There is no obvious reason why *Pisaster* could not feed most heavily in the winter. A few specimens of *Pisaster* are feeding in the winter in the field, and *Pisaster* feeds all winter in laboratory tanks (1°-1½° C. warmer than in the field). In addition, much of the prey of *Pisaster* is perennial and therefore occurs in the intertidal zone at all seasons.

However, *Pisaster* feeds most heavily in the summer; I will offer three hypotheses to explain why.

A. *Physiological specialization.* It could be argued that *Pisaster*'s metabolism functions most efficiently by limiting the processes that occur within it at any given time. It may be better to either feed, and process the components of the food, or, assemble these components into gametes. Perhaps the two processes are in some way mutually inhibitory. However, no other organisms, including starfish, have been shown to profit from the above mechanism, and some sea-stars do, in fact, carry on both processes simultaneously. Moreover, Figure 3 shows an increase in feeding before spawning.

B. *Subtle environmental changes.* Small changes in a number of environmental factors could adversely affect the efficiency of winter feeding. As a temperate sea-star, *Pisaster* must be able to withstand relatively high temperatures, 15–20° C., and, as expected, does show a lowered metabolic rate with lowered temperatures (Paine, personal communication). Thus, although the annual temperature range of sea water is not very drastic (6–13° C. at Lonesome Cove; 11–15° C. at Monterey, California), the seasonally slower digestion and locomotion may prevent *Pisaster* from effectively hunting in the intertidal zone during the limited period of a high tide. In addition, *Pisaster* left exposed by the ebbing tide would more likely be subjected to storms and freezing conditions in the winter. *Pisaster* seems resistant to heat; Feder (1956) reports they can withstand exposure to the summer sun for 3–6 hours and still appear "healthy, turgid, and moist." The higher summer feeding incidence would then result from *Pisaster* foraging higher in the intertidal, and hence encountering recently-set *Balanus* and *Mytilus*. After the consumption of these, and the seasonal onset of less favorable conditions, *Pisaster* migrates lower into the intertidal zone. Quantitatively less food, and temperature-inhibited locomotion, then produce the seasonal low in feeding intensity.

C. *Arm size limitation.* *Pisaster* is a very hard-bodied starfish; as an inhabitant of an area regularly exposed to violent wave action and desiccation, the evolution of this body strength is to be expected. *Pisaster* is too large to protect itself under rocks as, for example, *Leptasterias*, a smaller common intertidal starfish, usually does. This rigidity implies an approximately constant volume.

Several arguments suggest that a medium size is best for *Pisaster*. Growth studies (Quayle, 1954; Feder, 1956; Mauzey, unpublished data) suggest rapid growth up to reproductive maturity at about 150 grams, slower thereafter until about 400 grams, and very little or none at larger sizes. Large-sized starfish collected from Lonesome Cove do not seem to have proportionately larger gonads, suggesting that there is only small gain in growing above 400 grams, and that this energy might better be put into each year's gamete production. Furthermore, the volume, and therefore the metabolic demands, increase as the cube of linear dimensions; but the efficiency of hunting, since it seems to depend on either contact with the prey or chemoreception, would only increase with the surface area, the square of the linear dimension. The upper size limit is probably not set by the maximum size a starfish of a particular age can attain, but rather by an interaction with the size and abundance of prey in any particular area. A plentiful supply of large prey items must be available to meet the demands of very large *Pisaster*.

Since some coelomic space must be reserved for fluid, necessary for flexibility,

whatever arm space is taken up by pyloric caeca cannot be used for reproduction; the maximum number of gametes will be released if the pyloric caeca are smallest when the gonads are largest. Observations on lipid content in *Pisaster*'s organs further suggest that space may be a limiting factor. Lipids represent about twice the energy content per unit of weight of either proteins or carbohydrates. For ovaries, testes and pyloric caeca, the proportion of lipid is highest (30%, 18%, 50%, respectively) when the organ involved is largest, and lowest (5%, 2%, 30%) when the organ is smallest (Greenfield *et al.*, 1958).

For the reasons given under (B) above, feeding might be more efficient in the summer. *Pisaster* can reproduce most effectively if it does not feed in the winter, except minimally to replace metabolic use; calculations of the metabolic consumption of *Pisaster* (Paine, personal communication) indicate that the dry weight needed for maintenance at winter temperatures is about equal to that ingested by *Pisaster* in January and February (about 3 grams per one tidal cycle per 100 animals). According to Hypothesis (C), the sum of the gonad and hepatic indices should be fairly constant. This sum is plotted with respect to time in Figure 3. An approximately constant level is maintained from December through May. There is an abrupt drop in June, associated with spawning, and an eventual recovery to saturation level from July through November. This cycle is inverse to the feeding curve. The sum appears to be relatively constant at 13% to 17%, except upon spawning, before feeding has had time to build up the pyloric caeca again. Given a restricted structural framework, and the great advantage of a spring gamete release, the inverse gonad-pyloric caecum size is to be expected in *Pisaster*. Selection must favor the greater number of gametes produced with this strategy.

My study has benefited from the patience and stimulating discussion of Drs. Gordon H. Orians and Robert T. Paine. Dr. Robert Fernald has been very helpful in making available the facilities of the Friday Harbor Laboratories of the University of Washington. Financial support was provided in part by an NSF Marine Sciences Training Grant to the Friday Harbor Laboratories.

Mr. and Mrs. Roy Durlhack, the owners of the Lonesome Cove Resort, have been most gracious in allowing me to use their property, and very understanding of the requirements of my research.

SUMMARY

1. *Pisaster ochraceus* shows a definite seasonal feeding periodicity, in terms of per cent of the population feeding at one time, dry weight ingested, and in composition of ingested prey. Less than 5% are feeding in January and February; 60% to 80% in July and August. The dry weight ingested varies from about 3 grams per tidal cycle per 100 animals in the winter to about 30 grams in the summer months. Chitons are the principal winter prey, while barnacles and limpets are fed on most often in the summer.

2. Cyclic changes in gonad and pyloric caeca size and histological appearance characterize this species. The gonads are smallest in the fall, and grow during the winter to a maximum in the late spring, when spawning occurs. The pyloric caeca

size-changes are approximately inverse to those of the gonads. Seasonal histological changes of the oocytes, and storage granules in the pyloric caeca, are correlated with the gross organ patterns.

3. Two factors are suggested as explanations for these cyclic phenomena. (a) More favorable summer feeding for both the adult and larval *Pisaster* may have led to evolution of a storage function for the pyloric caeca; nutrients could then be transferred to the gonads in the winter. (b) It would seem evolutionarily advantageous to fill more of the limited space available in the arms with gonads than with pyloric caeca in the spring, at the time of spawning.

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SEQUELAE OF THE LD/50 X-RAY EXPOSURE OF THE PRE-IMPLANTATION MOUSE EMBRYO: DAYS 0.0 TO 5.0¹

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In a recent study by Rugh and Wohlfrohm in 1963 it was found that it took varying exposures of x-rays to kill *in utero* half of the mouse embryos of different ages, and in a still further study by the same investigators in 1965, pre-natal exposures and post-natal mortality data were presented. It was impossible to establish the LD/50/30 of x-rays for the mouse embryos of the first 5 days of gestation (since they were either killed *in utero* or appeared to be unaffected). It was therefore decided to use the LD/50 dose for the embryos *in utero* and study the sequelae exhibited by their survivors. In other words, doses from 100 r to 350 r were used which killed approximately half of the early mouse embryos *in utero*, and those which survived this radiation insult were examined during their lifetime for evidence of permanent but tolerable damage.

In a previous study by Rugh, Duhamel, Chandler and Varma in 1964 it had been shown that when the mouse embryos were exposed at the various gestation ages to the uniform dose of 100 r x-rays, there was variable response with respect to cataractogenesis. The variations in response were related to the gestation age at exposure. It was found that the highest incidence of cataract development, at 18 months of age, occurred when the mice were x-rayed at the time of fertilization or 0.0 days. Such mice developed 97% cataracts while the parallel controls showed 17% for the females and 13% for the males. The difference between those x-rayed and the controls represented at least 80%, which could be considered the incidence of radiation-induced cataracts. There was also a rather high incidence of cataracts among those x-irradiated during the next several days of gestation, so that it seemed in order to investigate this matter further.

This study includes effects of the LD/50 x-ray exposure of early mouse embryos in terms of litter size; sex ratios at birth and at 30 days; monthly weight variations; life span, blood, and skeletal changes and the etiology of cataracts.

MATERIALS AND METHOD

White female mice of the CF1 strain were put through a single pregnancy, using males of the same strain for mating, prior to their use in this experiment. For the experimental pregnancies the females were time-mated for two hours (8-10 AM) and those with vaginal plugs were segregated and marked as to the time of

¹ Based on work performed under Contract AT-(30-1)-2740 for the Atomic Energy Commission and aided in part by Grants RH-81 and RH-97 from the Division of Radiological Health, Bureau of State Services, U. S. Public Health Service.

conception. Some were x-rayed immediately (0.0 time) and others on the various days from 0.0 to 5.0 at which time implantation is in progress. Implantation actually begins at 4½ days but is a continuous process for several days prior to placenta formation. The dose of x-rays varied with the gestation age, being based upon a prior study (Rugh and Wohlfromm, 1963) in which the dose which would kill half of the embryos was determined. These doses are given in Table I in connection with litter size and sex ratios.

Upon delivery the mice were counted, sexed, anomalies recorded, and all were given to foster mothers who had not been x-rayed but who had had simultaneous litters. These foster mothers therefore provided normal post-natal care and nutrition until the time of weaning.

Those mice surviving at one month constituted the initial group for the selection of mice for this study. One hundred controls and 50 experimentals (half males and

TABLE I

This table gives the results of exposing early mouse embryos to the previously-determined LD/50 dose of x-rays, measured in terms of average litter size, sex ratios, and the survival of the respective sexes during their first 30 days of life. It demonstrates a drastic reduction in initial litter size, and (with the exception of gestation day 3 exposed to 140 r) a manifold increase in lethality during the first 30 post-natal days

Gestation age	LD/50 x-rays	Litter size average	Offspring at birth		Percentage lost in 30 days		
			Males	Females	Males	Females	Total
Controls	00	10.3	66	78	3.3%	3.8%	3.5%
0.0 gest. days	100 r	4.6	41	37	17.1	13.5	15.4
0.5 gest. days	275 r	6.7	45	48	11.1	12.5	11.8
1.0 gest. days	350 r	7.6	56	50	19.6	24.0	21.7
2.0 gest. days	125 r	6.1	44	42	11.5	9.5	10.4
3.0 gest. days	140 r	5.9	40	55	2.5	3.6	3.1
4.0 gest. days	330 r	6.5	48	73	37.5	13.7	23.1
5.0 gest. days	350 r	8.1	70	69	20.0	14.5	17.9
Totals (x-rayed)			344	374	17.0%	13.0%	14.8%

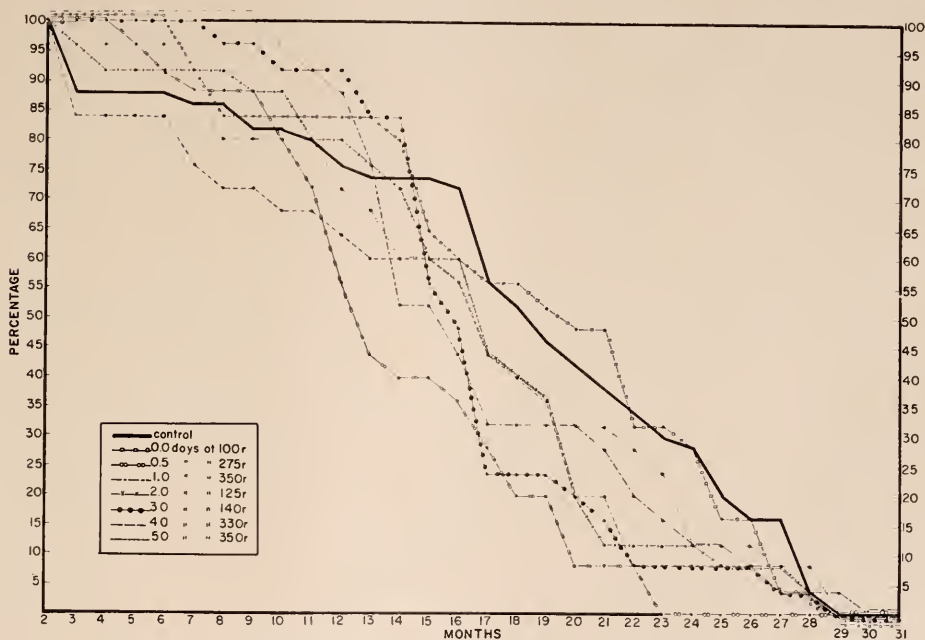
half females) were selected at random from those x-irradiated on each gestation day. Each mouse was earmarked for permanent identification and was examined and weighed each month through 31 months, or until none was left. At 2, 6, 9, 12, 18 and 24 months the eyes of every mouse in the series (2176 eyes) were examined by practicing ophthalmologists of this institution. The slit lamp was used to determine whether there were corneal opacities or incipient nuclear or cortical cataracts in the process of development. The pupils of the eyes were dilated at least one-half hour prior to the examination by a drop of 2.5% isopto-homatropine, used as a mydriatic. Thus, with each mouse identified by number and the condition of each eye recorded, it was possible to follow the onset and the development of cataracts in this study.

The x-ray facilities consisted of parallel tubes in cross fire spaced at 72 cm. each from the center of the gravid uterus. The machine was run at 184 KVP,

30 MA, with filters of 0.28 Cu and 0.50 Al, and having an HVL of 0.6 mm. Cu. The dose rate was 50 r/minute. The absorption from the plastic container and the scattering of radiations from the bodies of the mice balanced out so that the estimated dose absorbed by the embryos was very close to the air dose calculated at the position of the embryos described above.

EXPERIMENTAL DATA

The litter size at birth gives a fair indication as to the survival of embryos exposed to various doses and the several gestational ages. However, an accurate lethal



SURVIVAL OF MALE MICE : LD/50 X-RAYS FROM 0-5 DAYS POST-FERTILIZATION

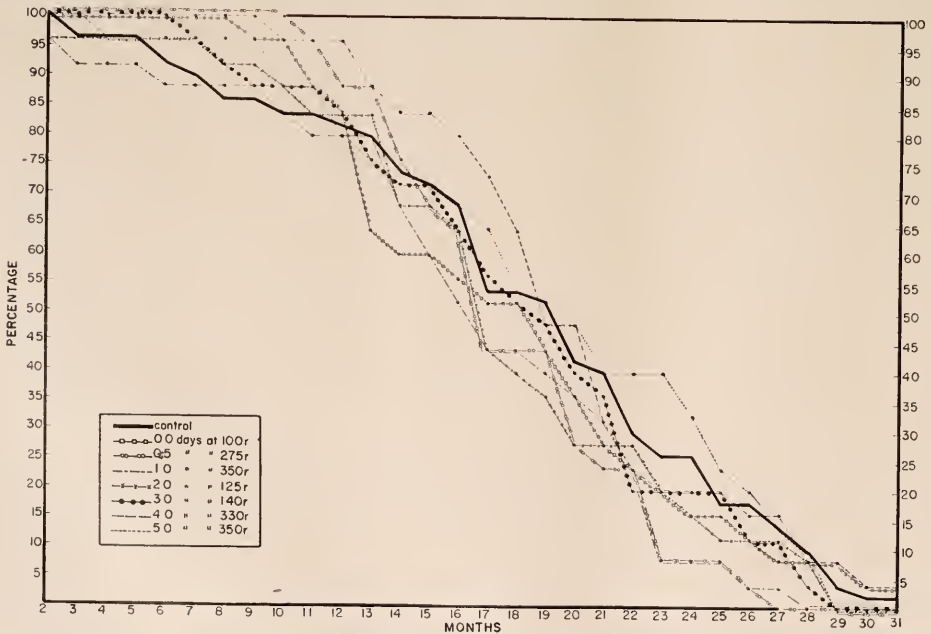
FIGURE 1. The dose of x-rays delivered to male mice on days designated varied because of the previous determination of the lethal dose to approximately half of such embryos. Nevertheless, the survival curve over the 31-month period did not vary much with the different gestational age exposures, although most were depressed slightly below the solid control line of survival. The most sensitive gestational age was 0.5 day after insemination, at which time early embryos received 275 r x-rays.

x-ray dose to half of the embryos (LD/50) for pre-implantation stages is rather difficult to establish without determining the number of viable eggs fertilized. This cannot be done without sacrifice of the animal. The average implantation number for this strain of mice was found to be between 11 and 12.

Among those mice which came to term there was a slightly higher number of females than of males, and during the first 30 days of their lives 17% of the males and 13% of the females died. In all cases except those x-rayed at 3.0 days to 140 r,

deaths during these first 30 days of life far exceeded the record for the controls, which was 3.5%. Since this study was based upon x-rayed mice which survived embryonic and fetal life, and also the first 30 days of post-natal life, and were studied throughout their life span, the data for weight, skeletal, blood, and cataract changes relate only to the hardiest of the mice exposed *in utero*. It must therefore be presumed that death of many mice deprived us of further statistical data relating to these physical variables.

The survival of mice selected at one month of age is shown for males and females separately in Figures 1 and 2 during the succeeding 30 months. Note the LD/50 exposures for the various gestation days which kill half of the embryos in utero. These figures (1 and 2) suggest that there is little permanent damage, in terms of life-shortening, when the experimental mice are compared with the controls. The controls are shown in heavy solid lines and those of the various gestation ages receiving different exposures are shown in the other curves. The experimental males did show a slight reduction in survival value, while the difference between the sexes was not at all pronounced in the controls. For the females (Fig. 2) the control curve cuts through the middle of all of the other experimental curves, indicating no effect. Thus, it is evident that those early mouse embryos which



SURVIVAL OF FEMALE MICE: LD/50 X-RAYS FROM 0-5 DAYS POST-FERTILIZATION

FIGURE 2. The dose of x-rays delivered to female mice on gestation days designated varied with the previously established lethal dose to half of the embryos. Here again the most radiosensitive gestational age, with respect to survival, was the embryo exposed at 0.5 day after insemination, with 275 r. However, the deviation from the solid control line was not statistically significant for either male or female mouse. In other words, those mice which survived x-irradiation *in utero* tended to survive as well as did the controls.

TABLE II

Average weights in grams of mice x-rayed in utero during early development

Day x-rayed LD/50	Average weight in grams			
	2 months	12 months	18 months	24 months
males	29.8	37.2	36.1	33.8
0.0 days				
females	23.9	28.3	31.2	30.8
males	29.1	35.2	33.8	0
0.5 days				
females	23.3	28.6	29.2	26.4
males	29.4	37.5	37.9	34.8
1.0 days				
females	22.7	30.7	32.8	29.8
males	30.8	40.2	37.1	33.4
2.0 days				
females	23.2	29.1	32.7	26.0
males	29.5	38.6	36.4	34.7
3.0 days				
females	24.3	29.2	32.2	29.9
males	29.7	36.6	35.8	34.5
4.0 days				
females	23.3	30.2	33.6	33.7
males	29.0	35.1	32.7	30.4
5.0 days				
females	23.5	28.3	29.2	25.9
males	27.8	36.2	36.3	34.5
Controls				
females	22.4	28.6	30.7	29.8

tolerate the x-ray exposures used, and survive at birth, and the first month of life thereafter, can be expected to survive almost as well as do the parallel controls.

The monthly weight records for each mouse are reduced to four periods (2, 12, 18 and 24 months) in Table II below. It can be seen that without exception, among the controls or the x-irradiated, the females of the same age were lighter in body weight than were the parallel males. It is also obvious that there was little, if any, statistical difference between the x-irradiated and the control mice by two months of age. The average weight for the first month of age is generally higher for the experimental mice than for the controls because of the reduced litter size of the experimentals and consequently more growing space for the remaining mice. Thus, again it seems evident that x-irradiation of the pre-implantation mouse embryo, if it survives the first post-natal month, will allow it to be as heavy as the controls.

The mice chosen at one month of age for the long term study were radiographed at two months in order to determine whether there was any evidence of permanent skeletal effects. Fifty male and 50 female controls were simultaneously examined, in the same manner as the various irradiated groups (each comprised of 25 males and 25 females). By direct comparison of such averages any contrast with the controls is obvious.

The mice were not anesthetized but were fastened to a plastic board by means of adhesive tape, and were radiographed at 40 volts, 10 MA, at 20 inches distance from

TABLE III
Skeletal measurements by radiography at 2 months of age (in cm.)

Gest. day	r	Sex	Tot. #	Skull		Spine	Humerus	Ulna	Femur	Tibia
				Lat.	A.P.					
Controls	0	M	50	1.05	1.44	6.11	1.20	1.39	1.47	1.68
		F	50	1.04	1.42	5.90	1.15	1.35	1.46	1.67
0.0	100	M	25	1.08	1.50	6.45	1.23	1.42	1.52	1.72
		F	25	1.09	1.46	6.08	1.19	1.38	1.49	1.71
0.5	275	M	25	1.08	1.47	6.21	1.21	1.40	1.51	1.70
		F	25	1.07	1.43	6.01	1.16	1.35	1.48	1.66
1.0	350	M	25	1.07	1.46	6.25	1.20	1.38	1.50	1.69
		F	24	1.05	1.44	5.95	1.14	1.35	1.46	1.68
2.0	125	M	25	1.10	1.50	6.22	1.22	1.42	1.53	1.74
		F	24	1.08	1.46	6.05	1.18	1.37	1.49	1.69
3.0	140	M	25	1.07	1.47	6.49	1.26	1.43	1.53	1.74
		F	25	1.06	1.44	6.28	1.20	1.39	1.52	1.71
4.0	350	M	23	1.05	1.46	6.35	1.23	1.40	1.51	1.71
		F	25	1.04	1.46	6.18	1.20	1.40	1.53	1.73
5.0	350	M	25	1.07	1.47	6.36	1.23	1.42	1.51	1.72
		F	24	1.06	1.45	6.23	1.20	1.39	1.52	1.74

the x-ray source, for two seconds, over sheet film. This amount of x-irradiation was regarded as inconsequential at two months of age. The radiographs were of sufficient clarity as to allow exact measurements of the skull (lateral and AP); spine, humerus, ulna, femur and tibia. Since the measurements were taken from a minimum of 23 x-irradiated mice, and 50 controls of each sex, giving a total of 345 experimental and 100 control radiographs, the data have statistical validity. Table III below gives the average measurement in centimeters for both sexes of each group of experimental and control animals.

It can be seen that the early x-irradiation of the mouse embryo had no adverse effect on the skeletal growth of the survivors, and in fact there appeared to be a tendency of those x-irradiated to be slightly larger than the controls. Again, it

must be pointed out that x-irradiated mice came from depleted litters so that the survivors had more room in which to grow, hence at birth and at one or two months they would be expected to be heavier and have larger skeletal parts than did the parallel controls. It was found by Rugh, Duhamel, Osborne and Varma in 1964 that mice x-rayed to 100 r at 12 to 14 days gestation showed serious defects in skeletal growth, and were all somewhat stunted, but these pre-implantation embryos, x-rayed from 100 r to 350 r, were unaffected with respect to the ultimate skeletal size.

Complete blood counts were made of both the control and x-rayed mice at 2, 6, 9, 12, 18 and 24 months of age. This included the usual determination of hemoglobin, white and red cell counts, platelets, and differentials based on 100 W.B.C.'s. The data do not deviate sufficiently from the controls to be presented in detail; suffice it to say that the x-irradiated mice tended to have slightly higher white cell counts and slightly lower red cell counts than the parallel controls. Whatever damage may have been produced in the mice from x-irradiation during the first five days of gestation was rectified by the time the survivors were two months of age.

As all mice progressed in age there was a drop in the hemoglobin and erythrocyte counts. Similarly a general trend was observed in the decrease of the number of lymphocytes and an increase in the neutrophils from two to 24 months of age. There was no evidence of leukemia in any of the mice examined. At 24 months four cases of lymphocytosis were found, one of which was a control. The leukocyte counts varied from 45,000 to 163,000 in these mice, of which an average of 89% were lymphocytes, all of a mature type. No further histological examination was provided for so that the exact nature of the lymphocytosis could not be determined. At 18 months the highest leukocyte count was 21,800 with no comparable lymphocytosis being noted.

A total of 1098 examinations of mice are presented for cataracts (2176 eyes) at 2, 12, 18 and 24 months of age. Examinations were also made at 6, 9 and 29 months but these data are not included in Table IV. Three major effects were recorded: corneal opacities, which are apparently minor abrasions of the cornea (conjunctiva) from which many eyes recover; nuclear sclerosis, which is a pre-cataract condition; and the distinct cataract. The corneal opacities seemed to be unrelated to the onset and development of cataracts while nuclear or even cortical sclerotic conditions of the lens almost always led to cataracts. Occasionally a corneal opacity obscured a lens in such a manner as to make the determination of a cataract difficult.

For the controls there were 50 males and 50 females at the beginning of the study, when the mice were one month of age. For each of the groups of mice x-irradiated at the various gestation days there were selected 25 males and 25 females, also at one month of age. These original numbers dropped steadily after 12 months of age so that by 24 months there were very few mice alive, either x-rayed or controls (Figs. 1 and 2). Thus the cataract data are derived as percentage data rather than actual numbers, and such percentages become less significant as the total number decreases (Table IV).

The incidence of cataracts at 18 months for the controls corresponds very well with the data of the previous study by Rugh, Duhamel, Chandler and Varma in 1964, but by 24 months even the controls showed an increasing incidence of

TABLE IV*
Cataract incidence following LD/50 x-ray exposure of mouse embryos fertilization to 5 days

	2 months						12 months						18 months						24 months				
	# Mice	% CO	% Scl.	% Cat.	% Tot. Cat.		# Mice	% CO	% Scl.	% Cat.	% Tot. Cat.		# Mice	% CO	% Scl.	% Cat.	% Tot. Cat.		# Mice	% CO	% Scl.	% Cat.	% Tot. Cat.
Controls	♂ 50 ♀ 50	44 50	0 0	0 0	0 0		38 41	43 45	1 9	5 6	6 15		26 27	38 50	1.9 7	12 7	14 14		13 13	38 27	8 12	31 54	39 66
0.0 day (100 r)	♂ 25 ♀ 25	50 52	2 0	0 0	2 0		21 21	17 21	0 5	36 33	36 38		14 13	14 19	4 8	39 54	43 62		7 5	21 20	0 0	36 100	36 100
0.5 day (275 r)	♂ 25 ♀ 25	24 56	0 0	0 0	0 0		14 22	4 2	7 2	0 20	7 22		5 11	10 5	20 0	0 41	20 41		0 2	0 25	0 0	0 25	0 25
1.0 day (350 r)	♂ 25 ♀ 24	66 67	0 0	0 0	0 0		22 20	7 5	0 2.5	18 15	18 18		8 11	31 5	0 27	19 14	19 41		3 2	0 0	0 0	50 0	50 0
2.0 days (125 r)	♂ 25 ♀ 24	56 54	0 0	0 0	0 0		20 21	50 45	5 5	5 14	10 19		11 11	45 45	27 9	18 14	45 23		2 4	50 50	0 0	50 25	50 25
3.0 days (140 r)	♂ 25 ♀ 25	50 56	0 0	0 0	0 0		23 21	20 10	4 2	15 19	19 21		5 13	20 4	20 0	20 8	40 8		2 5	0 0	0 30	50 40	50 70
4.0 days (330 r)	♂ 24 ♀ 25	58 46	0 0	0 0	0 0		16 24	34 15	0 17	6 4	6 21		10 12	35 29	10 17	10 17	20 34		2 5	0 0	0 2	100 60	100 80
5.0 days (350 r)	♂ 25 ♀ 24	32 40	0 2	0 0	0 2		18 21	28 21	0 7	14 19	14 26		11 13	32 8	9 19	14 23	23 32		3 9	0 6	0 6	66 56	66 62

* CO = corneal opacity, which was generally transient; Scl. = sclerosis, generally nuclear but occasionally cortical; Cat. = distinct cataract. Note that survivors at 18 months showed a higher incidence of cataracts among those x-rayed *in utero* than in the controls, but that at 24 months the data are irregular, because of the high mortality intervening.

cataracts (males 39% and females 66%). This suggests that such cataracts are truly senile cataracts, but the onset in this strain of mice appears to be slower than in some other strains.

It appears that homozygous mice can have congenital cataracts, while heterozygous mice tend to have normal vision. The onset of cataractogenesis in different strains may differ considerably. It is of interest to note that within any group of similar mice, similarly irradiated, cataract development is never all-or-none; there is a great variation in response (Gowen, 1962).

Among the x-irradiated mice surviving to 18 months, in almost every set of data it is obvious that those x-rayed had a higher incidence of cataracts than did the controls at the same age. However, when the incidence of cataracts among the few survivors at 24 months is determined, the range was from 0% to 100% among those x-rayed, as compared with an average of 53% for the controls. It must be recalled that mortality of many irradiated mice left only the most hardy ones to be included in this study.

SUMMARY AND CONCLUSIONS

1. Either x-rayed or control male mice had average weights in excess of the females at a comparable age. Pre-implantation mouse embryos, subjected to x-rays and surviving for 24 months, showed no gross adverse weight effects of the exposures. In some instances those with a radiation history were heavier, probably because they came from depleted litters which had more growing space within the uteri.

2. Whole blood counts indicated that mice x-irradiated in the pre-implantation stage tended to have slightly higher white cell counts and slightly lower red cell counts than their parallel controls. Otherwise any possible hematological damage appears to have been rectified.

3. There were no permanent skeletal effects on mice x-irradiated *in utero* during the pre-implantation stages of 0.0 to 5.0 days, as determined by radiographs of five selected bones and two skull measurements at 2 months of age.

4. Mice x-rayed at fertilization or at 5 days gestation showed almost as good survival as did the controls, but those x-rayed on days 1, 2, 3 and 4 showed slightly reduced survivals.

5. Corneal opacities occur frequently in these mice. Their eyes appear to be anesthetized to the particulate material in the bedding. There appeared to be no direct relationship between corneal opacities and the development of cataracts. Many mice with corneal opacities at two months recovered normal corneas at a later date.

6. Mouse cataracts appear to arise as nuclear or cortical sclerosis of the lens, but those arising in the nuclear region appear to be in the majority. The ultimate cataract, regardless of its origin, appeared to be similar in its involvement.

7. At any test period the percentage incidence of cataracts among the survivors was always higher among those x-rayed *in utero* than among the parallel controls.

8. Variations in cataractogenesis existed between males and females similarly x-rayed, as had also been shown in the previous study with the uniform exposure of 100 r x-rays. There appeared to be a sex differential in cataractogenesis of x-ray origin.

9. The fact that cataracts appeared earlier and to a greater extent among the x-irradiated mice than among the controls suggests that x-rays may hasten the onset of the usual senile cataracts.

10. There appears to be a greater incidence of bilateral as opposed to unilateral cataracts, and this seems to be particularly true for the females. The incidence of cataracts in one eye leading to bilateral cataracts occurred more frequently in mice x-rayed at fertilization and in females x-rayed at 1.0 and 5.0 days gestation. Thus, there was no clear-cut evidence of uni- leading to bilateral cataract development except possibly among some potential females. The precursors of the two eyes of any mouse at these early stages presumably received the same degree of radiation insult.

11. Since this study is based entirely upon x-irradiation of the early mouse embryo from fertilization to 5 days gestation, and since it has been demonstrated that x-rays during this period do in fact increase the incidence of cataracts, it must be presumed that the damage is done to the precursors of the lens since lens development is not initiated until about 11 days gestation. It is suggested that the etiology of these radiation-induced cataracts may be through an interference with the developmental process, originating with damage to chromosomes insufficient to be lethal.

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THE EFFECT OF HYPOPHYSECTOMY ON SODIUM METABOLISM OF THE GILL AND KIDNEY OF *FUNDULUS KANSAE*¹

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If a euryhaline teleost is to maintain a reasonably constant internal environment when in fresh water or in sea water, the regulatory mechanisms operating in one environment must be capable of altered function when the animal moves into the other environment. Thus, the gill must convert from a site of ion uptake to one of ion excretion, and the kidney, which functions primarily in excreting excess water in dilute environments, must reduce its function to a minimum in the other situation. The degree and rate at which a euryhaline teleost can accomplish such alterations will determine, in part, how rapidly and how successfully transfers from one environment to the other can be made.

It now seems well established that euryhaline teleosts can reduce urine flow markedly in sea water (Holmes, 1961; Stanley and Fleming, 1964a, 1964b; Sharratt *et al.*, 1964; Fleming and Stanley, 1965), and that the reduction is due in part to a reduction in glomerular filtration rate (Holmes and McBean, 1963; Sharratt *et al.*, 1964; Stanley and Fleming, 1964a; Fleming and Stanley, 1965), and to an increase in the tubular reabsorption of water (Sharratt *et al.*, 1964; Fleming and Stanley, 1965). Further, rates of chloride and sodium flux have been shown to increase several times where a euryhaline teleost is moved from fresh water to sea water (Mullins, 1950; Motais, 1961; Gordon, 1963; Motais and Maetz, 1964, 1965).

A few reports of measurements comparing sodium fluxes across the gill with renal sodium loss, have appeared (Maetz, 1963; Maetz *et al.*, 1964; Bourquet *et al.*, 1964; Motais and Maetz, 1965), but such measurements for a single species in fresh water, during the course of adjustment to sea water, and after several days in sea water, have not, to our knowledge, been reported.

We wish here to report the results of such studies, and to describe the effects of hypophysectomy.

MATERIALS AND METHODS

The euryhaline killifish, *Fundulus kansae*, was collected from a salt spring "Boonslick" located in Howard County, Mo. The routine handling to these animals, the preparation of sea water (1000 mOsm./kg.) and the techniques used for hypophysectomy have been described elsewhere (Fleming *et al.*, 1964; Stanley and Fleming, 1964b, 1966; Fleming and Stanley, 1965) and need not be repeated here. All experiments were carried out at $19 \pm 1^\circ \text{C.}$, and only females which

¹Supported by a NSF Cooperative Fellowship to the senior author and by NSF Grant GB-2264.

had been adapted to fresh water for at least two months were used. The fish selected all weighed approximately 2 grams.

Techniques for the collection and sampling of urine have also been described in detail elsewhere (Fleming and Stanley, 1965) and need only be summarized here. Urine was collected in a calibrated polyethylene cannula tied into the urogenital papilla. Urine volumes were estimated by reading directly from the calibration marks on the collection cannula. Figure 1 shows one of 10 separate compartments in an apparatus used to hold the fish relatively immobile during the experiment. A constant flow of 30 ml. per hour through each 24-ml. compartment was maintained by using a metering pump to remove water and a siphon from a constant-level reservoir to replace the water removed. Such an arrangement serves to

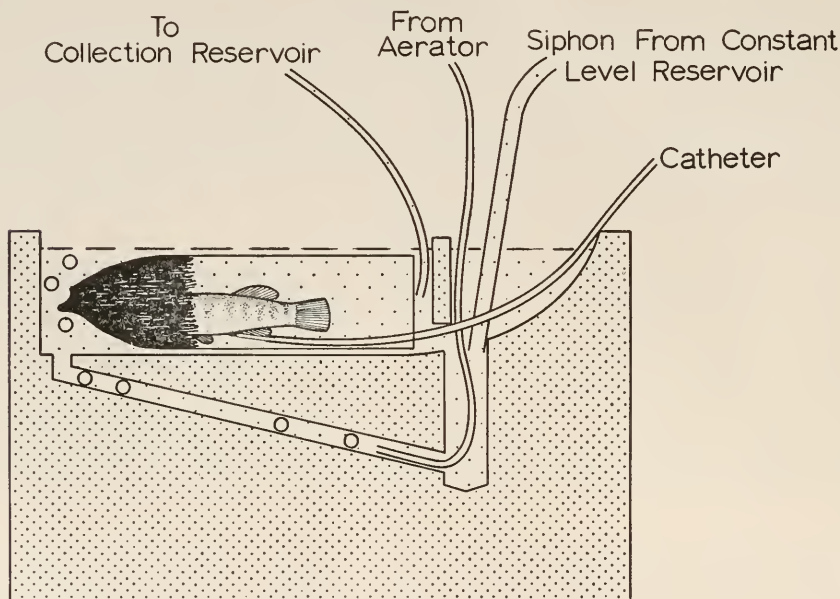


FIGURE 1. Cross-section through one of the units used to study the sodium metabolism of *F. kansae*. Urine sodium is collected in the catheter; that from other sites is carried to a collection reservoir.

separate kidney and gill excretion, and to provide a steady flow of water through the system, thereby reducing the possibility that any isotope excreted by the gill would be recycled. The water entering each compartment *via* the siphon was aerated, and each compartment was provided with a separate air line to further insure adequate aeration and to provide mixing. The water flowing through the chamber was collected in a collection reservoir.

As soon as a cannula had been secured, each fish was given an intraperitoneal injection of Na^{22} carried in fish Ringer's. Each animal received 4 microcuries of isotope carried in a volume of 7.5 microliters. A micrometer-driven syringe was used to control the volume injected.

At desired intervals, samples of urine and of the fluid bathing the gills were

taken. Urine was withdrawn from the collection cannula by carefully threading a length of polyethylene tube inside the collection cannula and applying gentle suction. Samples were removed every three hours in fresh water and every six hours in sea water. The entire quantity of urine produced for each time period was blown into three milliliters of 0.02% Sterox solution. The radioactivity of this sample was determined by the use of a deep well scintillation counter. The total urine sodium was then determined on the same sample by flame photometry and the specific activity of each sample calculated.

The collection reservoir was sampled, the volume measured, and the reservoir emptied every three hours. A 3-ml. sample was counted and the total radioactivity lost *via* the gill over the three-hour period determined by multiplying by one-third the volume (in milliliters) pumped through the chamber. In every case, the counting error was kept to within 3%.

As mentioned above, renal sodium loss was measured directly with flame photometry. The extra-renal (gill) sodium loss for any time interval was determined by the equation:

$$\text{Gill loss} = \frac{\text{Urine loss} \times \text{Total gill counts}}{\text{Total urine counts}}$$

The use of this equation is based on the assumption that the ratio: sodium-22/sodium-23, is identical for sodium lost *via* the kidney and *via* the gills.

Three separate types of experiments are reported. Experiments 1a and 1b were carried out using fresh-water-adapted animals that were cannulated, injected, and placed into fresh water. Twelve hours later, the animals were switched to sea water. Experiments 2a and 2b were carried out entirely in sea water, using animals that had been placed into sea water 8 days previously. In experiments 1a and 2a, only sham-operated animals were used; both sham-operated and hypophysectomized animals were studied in experiments 1b and 2b. In both sets of experiments, the behavior of the sham-operated animals was similar, and the data from these animals were combined. A total of 9 controls and 6 hypophysectomized animals were studied in fresh water and during the initial course of adjustment to sea water. Nine controls and seven hypophysectomized animals were examined in experiments 2a and 2b.

Experiment 3 compared the rate of sodium-22 uptake of sham-operated and hypophysectomized animals held in fresh water. A series of 8 flasks were set up, and 40 ml. of tap water containing Na^{22} were added to each flask. The isotope solution was such that each initial sample provided approximately 10^4 cpm. Two fish were weighed and placed into each flask. Enough additional solution was added so that the final volume was exactly 15 times the weight of the fish. Three-milliliter samples were withdrawn at each sampling period, counted, and returned to the flask.

RESULTS

Urine excretion

The changes in urine flow measured when *F. kansae* was transferred from a dilute environment into sea water were largely similar to those reported in an earlier paper (Stanley and Fleming, 1966); therefore, detailed data will not be

given here. Immediately prior to transfer, the controls were excreting urine at a rate of 330 ml./kg./day, and the hypophysectomized animals at a rate of 220 ml./kg./day. Both groups reduced urine flow to approximately the same value, *i.e.*, 20 ml./kg./day, within a few hours after transfer into sea water. The same levels of urine excretion were measured for both groups after an 8-day adaptation period to the saline environment. One difference was noted from the earlier

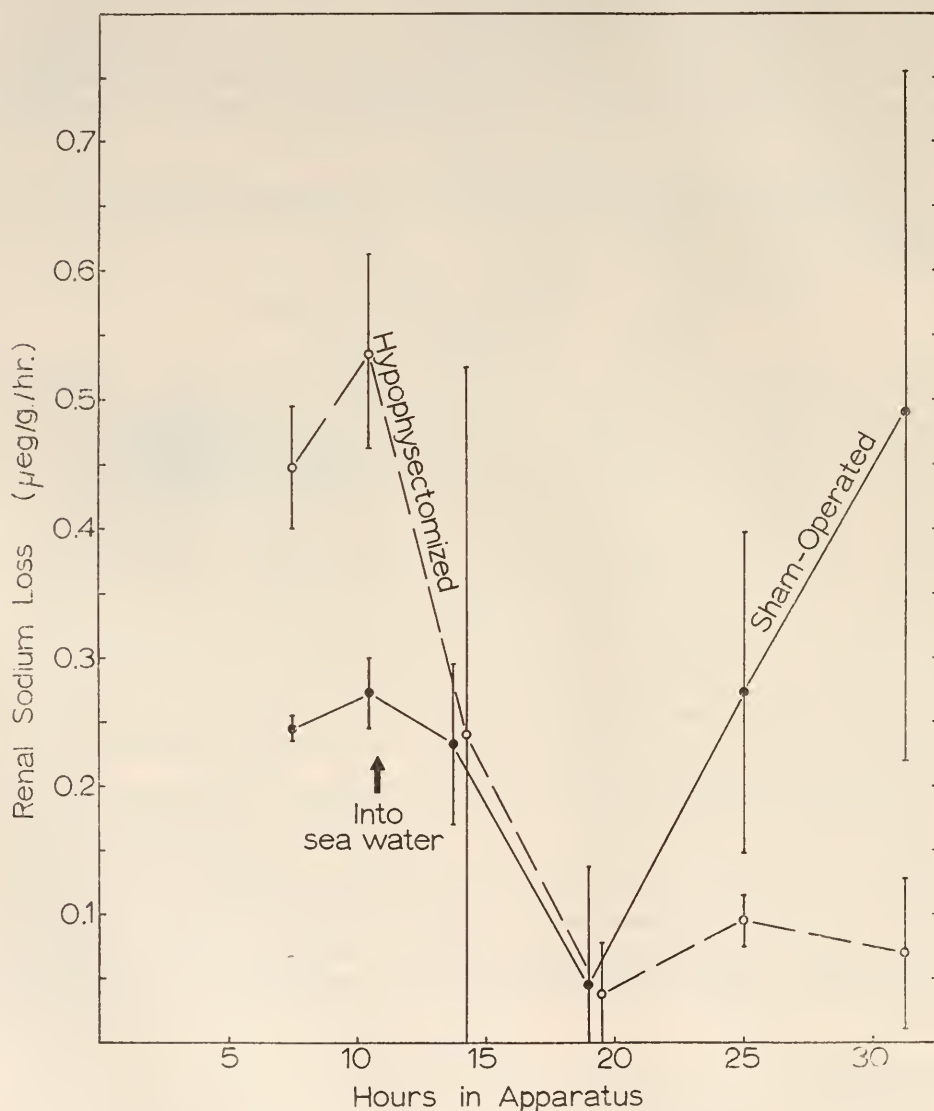


FIGURE 2. Comparison of renal sodium loss of sham-operated controls and hypophysectomized *F. kansae* held in fresh water and during the initial course of adjustment to sea water. Data show the mean $\pm$ S.E. for 9 control and 6 hypophysectomized animals.

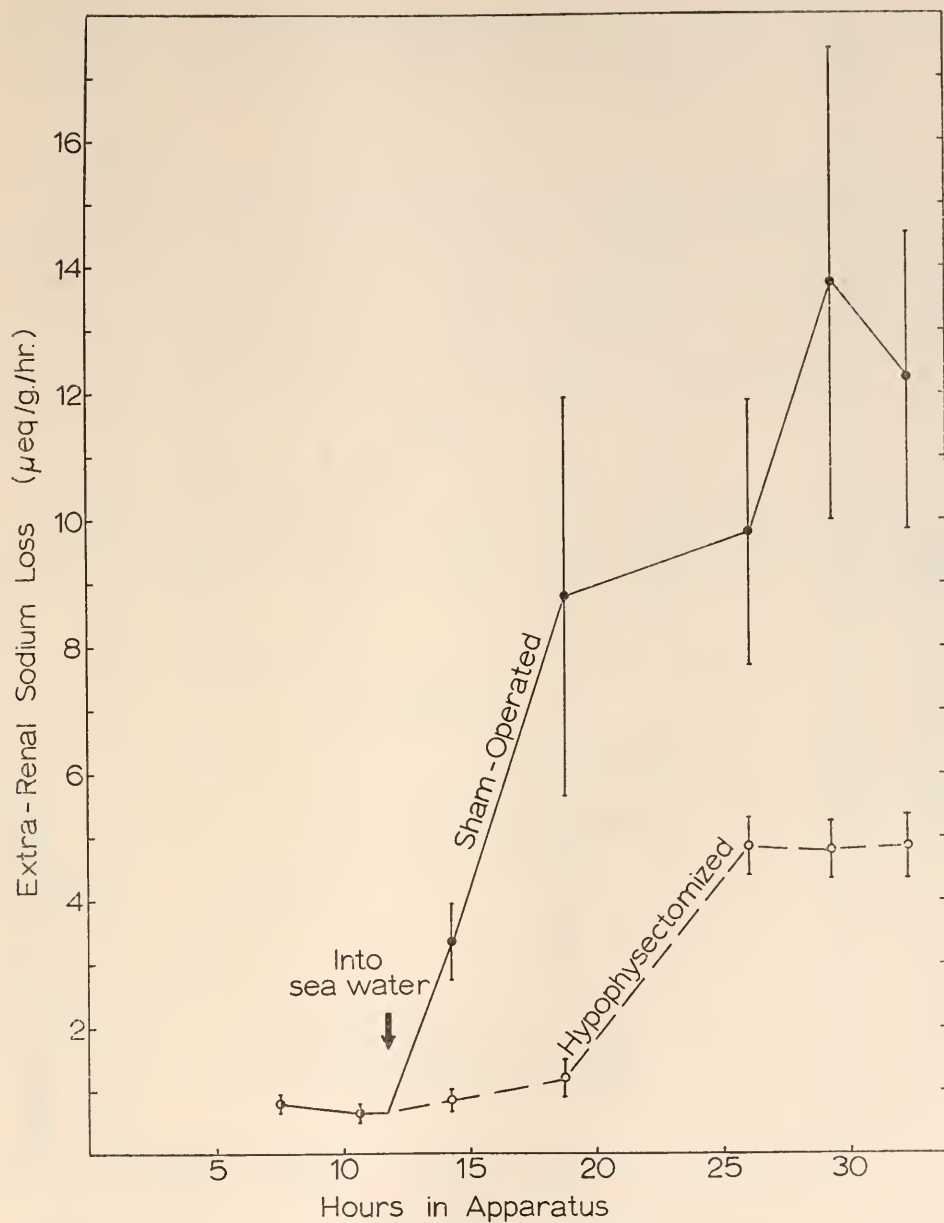


FIGURE 3. Comparison of extra-renal sodium loss of sham-operated controls and hypophysectomized *F. kansae* in fresh water and during the initial period of adjustment to sea water. Data show the mean $\pm$ S.E. for 9 control and 6 hypophysectomized animals.

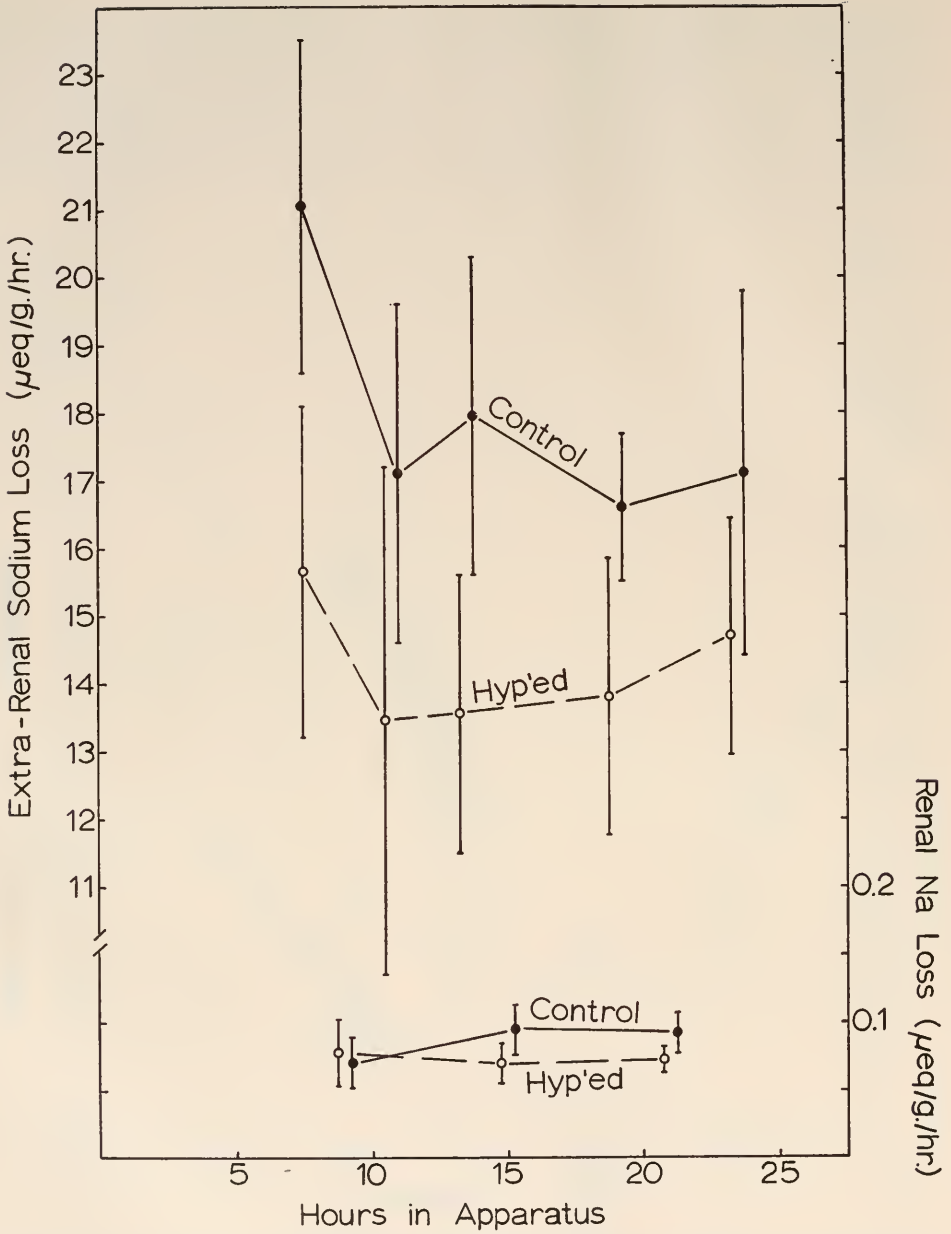


FIGURE 4. Renal and extra-renal sodium loss of sham-operated and hypophysectomized *F. kansae* after an 8-day exposure to sea water. Data show the mean $\pm$ S.E. for 9 control and 7 hypophysectomized animals.

experiments, *i.e.*, the hypophysectomized animals were able to reduce urine flow at essentially the same rate as the controls. Thus, hypophysectomy affects the rate of urine excretion of *F. kansae* in fresh water, but not in sea water.

Renal sodium loss

As shown in Figure 2, the renal sodium metabolism of the two groups differs markedly, both in fresh water and in sea water. Thus, the mean renal sodium loss of the control groups in fresh water was $0.27 \mu\text{eq./gm./hr.}$, for the hypophysectomized animals the mean figure was $0.53 \mu\text{eq./gm./hr.}$ Renal sodium loss fell to a low figure, $0.04 \mu\text{eq./gm./hr.}$ for both groups shortly after transfer into sea water and the hypophysectomized animals remained low there-after. The control groups showed a different response in that renal sodium loss soon increased, and by 20 hours after transfer had reached a mean value of $0.48 \mu\text{eq./gm./hr.}$

Extra-renal sodium loss

Contrary to the renal picture, hypophysectomy did not affect the extra-renal sodium loss of fish held in fresh water (Fig. 3). A marked difference was clearly evident, however, when the two groups were transferred into sea water. As shown in Figure 3, both groups showed a marked stimulation of sodium outflux

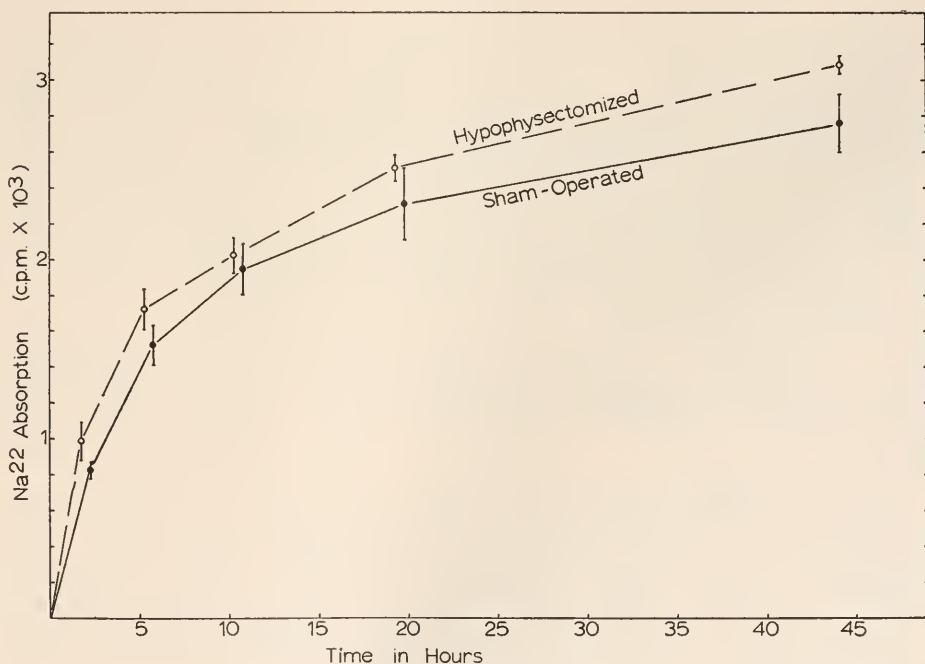


FIGURE 5. Sodium uptake of sham-operated and hypophysectomized *F. kansae* held in fresh water. Data show the counts per minute/ml. absorbed from a medium containing 10^4 cpm/ml. and 40 meq. Na/gm. of fish. Each point shows the mean $\pm$ S.E. of four pairs of fish.

after transfer, but the response shown by the hypophysectomized animals was considerably less than that shown by their controls.

Sodium loss after 8 days in sea water

Figure 4 compares both renal and extra-renal sodium loss of control and hypophysectomized animals after an 8-day adjustment period to sea water. Comparisons of Figures 2, 3, and 4 show that after 8 days in sea water, both groups of fish had the same low rates of renal sodium loss. On the other hand, extra-renal sodium loss had increased for both groups, with the hypophysectomized animals still showing somewhat lower values.

Sodium influx

Sodium influx is slightly higher for hypophysectomized animals than for their controls (Fig. 5). The disappearance of radioactivity from the environment is rapid at first and then levels off, presumably because of recycling of isotope. Influx in the controls for the first $5\frac{1}{2}$ hours was estimated by multiplying the fraction of radioactivity absorbed ($1/6.5$) by the sodium content of the medium ($40 \mu\text{eq./gm.}$ of fish) to give a value of $1.1 \mu\text{eq./gm./hr.}$ A similar estimate in hypophysectomized fish gives a value of $1.25 \mu\text{eq./gm./hr.}$

DISCUSSION

As pointed out elsewhere (Fleming and Stanley, 1965), the fact that *F. kansae* is a small fish means that a relatively large proportion of the body surface consists of water-permeable surfaces, *i.e.*, gills and oral membranes. Thus, a relatively copious urine flow, when compared with data on larger teleosts, is not surprising. A copious urine, however dilute, could provide a major site for sodium loss, and such is certainly the case for the plains killifish. Approximately 25% of the total sodium loss can be attributed to the renal route when this teleost is held in fresh water. *F. kansae* has no difficulty in remaining in sodium balance, however, and our estimates of sodium influx balance well with total sodium loss.

According to our estimates, *F. kansae* turns over approximately $1.0 \mu\text{eq. Na}^+/\text{gm./hr.}$ in fresh water. This figure contrasts sharply with that estimated from animals that had been exposed to sea water for 8 days. Under such conditions, renal sodium loss is negligible (0.6% of the total), and sodium influx would approximate outflux, *i.e.*, $17 \mu\text{eq./gm./hr.}$ —a 17-fold increase over the values estimated in fresh water.

It is also possible to estimate gill influx for the first few hours after transfer. During the first 12 hours in sea water, gill outflux averaged $7 \mu\text{eq./gm./hr.}$ During this same period, total body sodium rose from 62 to 132 $\mu\text{eq./kg. body weight}$ (Stanley and Fleming, 1965), an average of $6 \mu\text{eq./gm./hr.}$ The net increase in body sodium plus the outflux provides an estimate of sodium influx, *i.e.*, $13 \mu\text{eq./gm./hr.}$ for the first 12 hours in sea water, which is slightly more than a 13-fold increase in sodium influx over the fresh-water value.

Comparisons, then, of the estimates of sodium influx show a 13-fold increase over the fresh-water value for the first 12 hours in saline, and a 17-fold increase

for those fish held in sea water for 8 days. Sodium excretion, on the other hand, was only 7-fold higher during the first 12 hours after transfer, in contrast to the 17-fold increase estimated for the 8-day fish. These figures indicate that total body sodium must rise following a transfer to sea water, and indeed it does (Stanley and Fleming, 1965).

Both target organs respond promptly to the transfer into sea water, but the nature of the response is somewhat different. Thus, the kidney response was diphasic, sodium loss first falling from $0.28 \mu\text{eq./gm./hr.}$ to $0.04 \mu\text{eq./gm./hr.}$, and then rising sharply to $0.49 \mu\text{eq./gm./hr.}$ 20 hours after entering the saline environment. Unfortunately, the rapid loss of isotope in sea water made it impractical to continue these experiments for a longer period. It seems not unlikely that this figure would continue to increase, since this teleost can excrete a blood-hypertonic urine for a limited time (Stanley and Fleming, 1964b; Fleming and Stanley, 1965). While a figure of $0.5 \mu\text{eq./gm./hr.}$ may seem low, it is, nevertheless, sufficient to remove nearly 10% of the total body sodium over a 24-hour period. The low rate of renal sodium loss in animals immediately after transfer and in sea water for eight days can be ascribed to a low rate of urine formation.

It should be pointed out that any measurement of flux includes an error equal to exchange diffusion. In the present experiments, it is possible to place an upper limit on the magnitude of this error. Exchange diffusion should be approximately equal for all animals in sea water regardless of previous history. Exchange diffusion would then be less than the lowest outflux measurement, *viz.*, less than $5.0 \mu\text{eq./gm./hr.}$ as measured for hypophysectomized fish after initial adjustment to sea water (Fig. 3).

Previous experiments (Stanley and Fleming, 1966) have suggested a negative sodium balance for hypophysectomized *F. kansae* held in fresh water, *i.e.*, such animals had significantly less total-body sodium than did their controls. The data presented here suggest a negative sodium balance after hypophysectomy, and localize the metabolic fault at the kidney level. Thus, no differences in extra-renal sodium loss were observed, and the hypophysectomized fish took up sodium at a slightly higher rate than did their controls. The increase in influx, however, is not sufficient to compensate for renal loss, *i.e.*, 0.54 *vs.* $0.27 \mu\text{eq./gm./hr.}$ Although hypophysectomized killifish will live for several weeks in tap-water without food, it is necessary to provide additional sodium in their diet if they are to be held for any extended period. We have held hypophysectomized animals in fresh water for several months without difficulty, by feeding a commercial fish chow supplemented several times each week by frozen brine shrimp.

A comparison of Figures 3 and 4 suggests that hypophysectomy also affects the sodium metabolism of the gill, at least during the course of initial adjustment to sea water, *i.e.*, sodium outflux does not increase at the rapid rate shown by the control animals. After 8 days in sea water, extra-renal sodium loss is still 20% lower than in controls (Fig. 4). Hypophysectomy also affects kidney function during adjustment to sea water, *i.e.*, hypophysectomized fish do not produce hypertonic urine and do not show any increase in renal sodium loss following transfer (Fig. 2). Renal function is similar in the two groups after 8 days in sea water (Fig. 4). Thus, hypophysectomized fish appear to be less efficient in adjusting to sea water because both gill and kidney function are altered, but are capable of

sea-water-adaptation and by 8 days there are no significant differences in renal or extra-renal sodium metabolism between the two groups.

It has long been known that the European eel (*Anquilla anquilla* L.) can survive in fresh water after hypophysectomy (Fontaine *et al.*, 1949), and several studies dealing with the effect of such treatment on the sodium metabolism of this teleost have appeared recently (Chester Jones and Bellamy, 1964; Leloup-Hatey, 1964; Chester Jones and Henderson, 1965; Chester Jones *et al.*, 1965). Contrary to the data reported here for *F. kansae*, it appears that the eel remains in relatively close sodium balance after hypophysectomy, for such animals can survive in distilled water for some time—an environment that the plains killifish cannot tolerate for more than a few days at best (Pickford *et al.*, 1966). The eel also shows a marked drop in urine flow after hypophysectomy but urine sodium levels are not affected, *i.e.*, renal sodium loss is actually reduced. In contrast to *F. kansae*, the animals remain in sodium balance by reducing sodium uptake. Such data do not imply that electrolyte metabolism has not been affected, and it is clear that such is not the case, for hypophysectomized eels held in fresh water do show a drop in serum electrolytes (Leloup-Hatey, 1964; Chester Jones and Henderson, 1965; Chester Jones *et al.*, 1965).

SUMMARY

1. Renal and extra-renal sodium loss was measured for intact and hypophysectomized *Fundulus kansae* in fresh water and during adaptation to sea water.

2. In fresh water, urine was copious and dilute but a major route of sodium loss.

3. Following transfer to sea water, urine flow was reduced and extra-renal sodium excretion increased. Renal sodium loss decreased (because of a reduction in urine flow), then increased to above fresh water values, then, after several days in sea water, returned to a low value.

4. Hypophysectomized fish in fresh water had a reduced urine flow, an increased renal sodium loss, while extra-renal sodium outflux was unaffected.

5. Following transfer to sea water, hypophysectomized fish shut-down urine flow and although they increased extra-renal sodium excretion, they did not do so as rapidly as controls. Urine sodium loss was reduced and remained low.

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THE EFFECTS OF GLYCEROL AND OTHER ORGANIC SOLUTES ON THE MOTILITY AND RESPIRATION OF SOME INVERTEBRATE SPERMATOOA¹

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Glycerol, in high concentrations in aqueous solution, has been used for such varied purposes as preparing ATP-sensitive contractile systems and as a preventive of freeze damage to living cells. Added to sea water in 13% v/v concentration, some sperm of marine invertebrates maintain a vibratile activity (Steinbach and Dunham, 1961). Since there were indications that the glycerol did penetrate the cells, it was clear that the motile mechanisms were not completely impaired at a total osmolar concentration of solute of at least 2.5 osmolar.

The observations reported in this paper were made with the hope that a study of the effects of other organic solutes might reveal clues about the possible role of water structure in the motile mechanism. Regretfully it must be admitted that this hope was not realized but the studies did show certain physiological differences in the actions of the solutes chosen and species differences in the responses of the cells.

MATERIALS AND METHODS

Methods for collecting and washing *Arbacia punctulata* and *Mytilus edulis* sperm have been described (Steinbach and Dunham, 1961). The effects of the several agents appeared to be uninfluenced by the number of washes of the sperm. Most of the work involved sperm centrifuged down once from sea-water suspension and then resuspended in normal filtered sea water. All operations were conducted at room temperature in an air conditioned room 23–25° C.

For studies of motility, appropriate concentrations of the various agents were placed in small petri dishes and concentrated sperm suspension added in fixed amounts. A variety of methods for quantitative measurement of motility were tested without success. The results reported are hence subjective estimates, a – sign designated less motility than the sea water, $\pm$ = about the same as controls and + . . . varying degrees of activation.

Oxygen consumption was measured in Scholander (1950) differential volumeters operated in a large tank of water at room temperature. The volumeters were shaken with a low amplitude oscillatory movement at 40 times per minute. No extensive study was made of effects of rate or amplitude of shaking other than checking that rate of volume decrease was a function of the respiring mass.

¹ Work reported in this communication supported by grants from U. S. Public Health Service #GM 10542 and from the Wallace G. and Clara A. Abbott Fund of the University of Chicago.

In this report, respiration is given as per cent of that of sea water controls. Most of the published literature reports oxygen consumption of sperm based on units of 10^8 individual cells. Since it is not only laborious but subject to considerable variation to determine sperm numbers on each sample, we measured wet weight of pellet of a centrifuged sample of sperm suspension and referred oxygen consumption to units of wet weight determined in that fashion. Pellet weights were determined by pipetting measured volumes of sperm suspension into previously weighed 12-ml. conical centrifuge tubes. The tubes were then centrifuged

TABLE I

Data relating to some common organic solvents, their physical characteristics, ability to protect against freeze damage and to activate Arbacia sperm, motility or respiration

	Protection ^a erythrocytes	Arbacia sperm activation	Change viscosity ^b	HS ^c	Surface tension ^d	μ^e	ϵ^f	v.p. ^g
Glycerol	+++	± or —	20	~5/6	63		42	—
Ethylene glycol	+++	++	13	4/4	60?	2.3	37	—
DMSO	+++	+++	17	4/4	43	3.9	49	0.7
DMF	+++	+++	17	3/5	35	3.8	27	3.7
DMAC	+++	+++	n.t.	3/6	32?	3.8	38	1.3
Ethyl alcohol	±?	+	14	1/3	22	1.7	24	++
	++							
Methyl alcohol	+?	n.t.	6	2/2	23	1.7	34	++
Acetone	—	n.t.	7	0/3	26	2.9		++
H ₂ O			0		74	1.8	80	17

^a Estimated from different sources. Protection is reported differently depending on the freezing temperature used. Cf. Nash, 1962; Lovelock, 1954.

^b Increase in viscosity on dissolving 1 M/L. of substance in water. Approximate measurements with Ostwald viscosimeter.

^c Hydrophilic strength from Nash, 1962.

^d Surface tension of pure liquids. Figures estimated from published tables and technical literature. Those figures with question mark are estimated from lowering of surface tension of 1 M solutions.

^e Dipole moments. From technical literature and handbooks.

^f Dielectric constants. From technical literature and handbooks.

^g Vapor pressure. From technical literature and handbooks.

For the most part, figures given above are rounded out from more exact figures in the source material. For DMF and DMAC, Dupont has a Review of Catalytic and Synthetic Applications for DMF and DMAC. Crown-Zellerbach publishes a technical review of properties of DMSO (dimethyl sulfoxide. Reaction Medium and Reactant).

15 minutes at 3,400 rpm., 10 cm. radius to center of tube. The supernatants were then decanted, the sides wiped dry with tissue and the tubes weighed. In some instances dry weights were also determined.

The solutes are indicated in Table I. They were chosen primarily on the basis of previous studies of solutes conferring protection against freeze damage to cells (cf. Lovelock, 1959; Nash, 1962). They all fall into the general category of weakly protic (alcohols, etc.) or aprotic solvents (cf. Singer, 1962; Parker, 1960). All are polar compounds of relatively high dielectric constants. All could form

associations with water or other molecules by accepting protons but would be poor proton donors.

RESULTS

Tables I and II summarize the major results of observations on *Arbacia* sperm. *Arbacia* sperm show increased motility in the presence of high concentrations of DMF, DMSO, ethylene glycol and ethyl alcohol. At comparable concentrations glycerol has little effect or inhibits, while urea, inorganic salts and hexose sugars (not shown in table) stop motility and inhibit respiration. For DMF, the

TABLE II

Relative rates of oxygen consumption. *Arbacia* sperm; 2 ml. suspension per flask. Total dry weight of sperm per flask on the order of 50–80 mg. Reagents added as 0.25 ml. fluid per flask to give final concentrations noted. For each reagent, first value of relative Q_{O_2}

$\left(\frac{Q_{O_2} \text{ experimental} \times 100}{Q_{O_2} \text{ sea water control}} \right)$ is for the rate during the first 60 minutes.

Figures in parentheses indicate relative rates two or more hours later. + or – symbols indicate activity of sperm (see text)

Reagent added	Final conc. of reagent in sea water		
	0.09	0.45	0.9
DMF	170 (130) ++	580 (270) +++	340 (130) +
DMSO		225 (170) +++	
Ethylene glycol	125 (125) ±	125 (125) +	210 (150) +
Glycerol		67 ±	75 —
Urea	87 (57) —	57 (57) —	48 (48) —

optimum concentration for enhancement of motility and respiration is near 0.5 *M* in sea water but the effects are still pronounced in 1 *M* concentrations. In 2 *M* concentrations, stimulation is observed for a short time followed by irreversible depression of motility. Respiration was not measured at 2 *M* concentrations.

The figures in Table II are given as experimental rates expressed as percentage of Q_{O_2} values for sea water controls. For both *Arbacia* and *Mytilus* sperm the usual dose per flask was 50–80 mg. dry weight of sperm. Under such conditions *Arbacia* sperm used oxygen at a rate of ca. 2.2 $\mu\text{l.}^3 \text{ O}_2/\text{mg. dry weight/hour}$, *Mytilus*, ca. 1 $\mu\text{l.}^3 \text{ O}_2/\text{mg. dry weight/hour}$.

Approximately the same series of experiments was carried out with *Mytilus* sperm but the results are not presented in detail since all substances at the lower concentrations had little effect on either motility or respiration while at the higher concentrations both motility and respiration were depressed. The difference between the responses of the sperm from the different species was clear-cut and invariable even though morphologically the two cell types are rather similar.

Respiration of *Arbacia* sperm may continue at a fairly constant level for hours but more typically declines with time (Table II). Figure 1 gives the results of

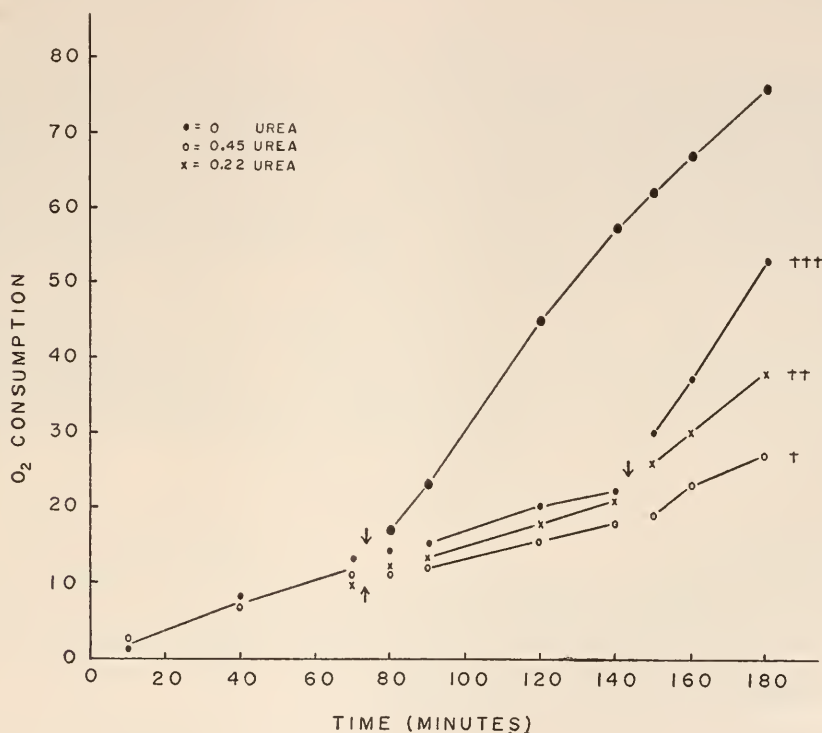


FIGURE 1. Oxygen consumption of *Arbacia* sperm (movement in mm. of plunger of volumeter) plotted against time. For the first 70 minutes, sperm were in normal sea water. At 70 minutes urea was added ($\uparrow$) to three of the volumeters (lower curves) with DMF, 0.5 *M* final concentration ($\downarrow$), added to volumeter of upper curve. At 140 minutes DMF 0.5 *M* added to volumeters of three lower curves. + indicates estimated motility of sperm.

one run in which additions of DMF (or sea water for control) to urea-treated sperm, stimulated respiration, even made at 70 minutes in the urea concentrations indicated. While no respiration experiments were carried out over very long time periods, the enhancement of motility by the 0.5 *M* concentrations of the substances was evident at least as long as 8 hours after the start of the treatment. It should be noted that the stimulating action of DMF, and presumably the other activating substances, is found with sperm held in sea water until respiration has dropped to a marked extent.

Observations, not reported in detail, were made of the effects of KCl, NaCl, sucrose and glucose on motility and respiration. At 0.5 osmolar in sea water and higher concentrations, motility and respiration were irreversibly depressed in *Arbacia* and motility in *Mytilus*.

While an extensive study of retention of fertilizing capacity of sperm was not attempted, a few tests showed that the ability of *Arbacia* sperm to activate *Arbacia* eggs was retained for several hours in the stimulating concentrations of DMF. There were, however, indications that the sea-water controls fared better in this respect.

DISCUSSION

At present there is no good explanation for the differences in the effects of DMF and related compounds on *Arbacia* and on *Mytilus* sperm. The morphology of the two cell types is rather similar and preliminary studies show no marked effects of DMF on the fine structure of either. A quick survey showed that DMF in 0.5 *M* concentration also activated *Phascolosoma* sperm but inhibited the sperm of *Loligo* and *Busycon*. Thus there are clear-cut comparative differences which may, in the future, assist in determining the nature of the effects of the organic substances on the motile mechanisms involved.

Focussing attention for the moment on *Arbacia* sperm, the cell type most extensively studied here, there is a parallelism between the stimulating effects on the sperm and freeze protection of human erythrocytes. This parallelism, together with some physical characteristics of the solvents, is noted in Table I.

On the basis of Table I and related data, the following criteria could be listed for either freeze protection or *Arbacia* sperm activation:

1. Substances effective are weakly protic polar compounds of fairly high dielectric constants.
2. Substances interact with water or form complexes in some fashion, as suggested by the increase in viscosities in aqueous solution.
3. Substances in pure liquid form have relatively high surface tensions (*ca.* 50% of water or higher) and vapor pressures lower than water.
4. Substances should penetrate cells readily.

The last-named requirement of ready penetration places glycerol in a sort of gray-area so far as effectiveness is concerned. Glycerol (and even glucose) is known to penetrate some cells rapidly, others very slowly. In contrast, there is every likelihood that the DMF-, DMSO-type substances penetrate virtually all cell types at rates comparable to that of water. The requirement that substances be not overly surface-active (number 3) appears to distinguish some of the weakly protic alcohols that confer only moderate protection, followed by irreversible change, from the most effective substances. The correlation between protective ability towards freeze damage and hydrophilic strength (HS), as pointed out by Nash, 1962, is noted in Table I showing also the characteristic that the substances of high HS values do not serve as good protective agents if the surface tensions of the pure substances are low. While not entirely demonstrated, it seems probable that those substances interacting strongly with water (viscosity increase, high HS values, etc.) but which are also surface-active, are not good protective agents

because of side deleterious effects, not because of lack of effects similar to those listed as good protective agents.

At the present time there appears to be no single set of characteristics common to the various substances which might explain their effects other than the interaction with water. In general, the reagents used might act on biological systems either by providing a mixed solvent of characteristics different from that of water or by having specific chemical interactions. At the present state of our knowledge it seems most reasonable to assume that the reagents listed in Table I act by altering the solvent properties of the system.

Weakly protic and aprotic substances, as pure solvents, do alter macromolecular structures (*cf.*, Singer, 1962) although most of the effects are evident only in high concentrations or in pure organic solvent. Effects on enzymes show up in general only in concentrations somewhat higher than those used in this paper (*cf.* Hamaguchi, 1964). Similarly antigen-antibody interactions appear little influenced at concentrations below 2 *M* (Gould *et al.*, 1964). On the other hand, ethanol in relatively low concentrations inhibits ion transport in animal tissues (Israel-Jacard and Kalant, 1965) and several solvents have a pronounced enhancing effect on relaxation of glycerinated muscle fibers (Watanabe and Maruyama, 1964). The aprotic solvents themselves (*e.g.* DMF, DMSO) alter ionic mobility relationships markedly when used as pure solvents (*cf.* Parker, 1962).

This brief summary of representative effects of various organic solvents does indicate that the influence of the agents on biological processes probably reflects rather delicate alterations in the functional machinery of the cell, rather than by participating as reactants in the metabolic systems.

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UPTAKE OF ORGANIC MATERIAL BY AQUATIC INVERTEBRATES.

IV. THE INFLUENCE OF SALINITY ON THE UPTAKE OF AMINO ACIDS BY THE BRITTLE STAR, *OPHIACTIS ARENOSA*¹

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The ability to remove amino acids and other small organic compounds from dilute solution is widespread among marine invertebrates. Stephens and Schinske (1961) reported examples from ten different phyla. This capacity has been studied in additional forms and has provided material for a series of reports (Stephens, 1962, 1963, 1964; Stephens *et al.*, 1965; Virkar, 1963). It has been our experience that any soft-bodied marine invertebrate exposed to an amino acid such as glycine or phenylalanine at concentrations ranging between 10^{-5} and 10^{-6} moles per liter shows the capacity to remove it from solution quite rapidly. The fresh-water forms we have examined remove amino acids from solution very much more slowly, so slowly that we have not demonstrated the occurrence of the process unambiguously.

The relation between external salinity and the uptake of amino acids has proved to be of interest. Stephens (1964) showed that uptake of glycine in euryhaline nereid polychaetes occurred only at moderate to high salinities. At lower salinities, uptake stopped almost entirely. The salinity at which uptake ceased was closely correlated with that at which osmoregulation and chloride regulation began. The data did not permit firm conclusions about rates of uptake at intermediate salinities since they were acquired before recognizing the considerable capacity for adaptation in the system.

A related matter of interest is the regulation of the "free amino acid pool" in marine invertebrates in response to changes in salinity. The tissues and body fluids of most marine invertebrates are in osmotic equilibrium with their environment. Numerous workers have reported large amounts of non-protein nitrogenous substances in the tissues, of which amino acids are the most abundant (see Awapara, 1962; Kittredge *et al.*, 1962). This pool of amino acids is sufficiently concentrated to represent a major fraction of the osmotic concentration of the tissues. It has been shown that as salinity is decreased, the size of the pool decreases. Florkin (1962) has suggested that this behavior represents an osmoregulatory response in the sense that decreasing the size of the free amino acid pool spares larger fluctuations in other cellular constituents. This position is supported by a large number of observations carried out by Florkin and coworkers (reviewed in Florkin, 1962), as well as observations by Potts (1958), Shaw (1958), and Lange (1963, 1964). Virkar (1963, 1965) has studied the response of tissues of the sipunculid *Golfingia* to small changes in salinity. The change in free amino acids in the body wall which is produced by lowering the concentration of the ambient medium by 10% is

¹ This work was supported by Grant GM 12889 from the USPHS.

large enough to account completely for the implied change in intracellular osmotic concentration.

The preceding paragraphs set a context in which several interesting questions can be asked. What is the source of free amino acids which form such a surprisingly concentrated pool in many marine organisms? Awapara (1962) argues it is not dietary, on the ground that there are differences in animals found in comparable habitats, but he does not make positive suggestions. By what means is the free amino acid pool in the tissues decreased in response to lowered salinity? We have no information whatever on this point. Does the free amino acid pool contribute significantly to the energy metabolism of the organism? What is the turnover rate of individual constituent acids in the pool?

The earlier work in our laboratory to which we have alluded is based on supplying uniformly labelled compounds to marine invertebrates in very dilute solution in the ambient medium. This provides a technique for labelling specific constituents of the free amino acid pool at will. The work to be reported uses this technique to provide data relevant to the questions raised above concerning the role of free amino acids in marine organisms. A portion of this work has appeared in abstract form (Stephens and Virkar, 1965).

MATERIAL AND METHODS

Ophiactis arenosa is a small brittle star which lives in close association with several sponges found on floating docks and on pilings. Animals were collected as required from Newport Bay south of Los Angeles. Masses of sponge were brought into the laboratory and kept in sea water. The brittle stars emerged on the surface of the sponge mass in about an hour and were placed in sea water in dish pans. Several hundred animals were kept in a single pan in an incubator at a temperature of 15–16° C. Observations were carried out at room temperature (about 21° C.). Individuals used in the observations reported were selected in the size range of 10 to 30 mg. wet weight except for the data concerning the relation between weight and rate of uptake of glycine.

These animals may be exposed to moderate salinity variations in their normal environment but presumably do not suffer rapid changes. However, they proved capable of surviving a direct change from full-strength sea water to 60% sea water. Acclimation to 60% sea water was necessary for survival at 50% sea water. One set of observations is based on the responses of organisms transferred abruptly to 60% sea water. Aside from this, animals were allowed to adapt by placing them one day at 90%, 80%, and 70% sea water successively. They were kept for two days at 60% and 50% sea water. Observations were undertaken after all animals had been acclimated in this fashion. Dilutions of sea water were prepared with distilled water. The salinity of the sea water stock was 33.08‰.

Water content was determined by weighing individuals after drying on filter paper and reweighing them after approximately 24 hours at 110° C. Amino acid determinations were carried out by measuring ninhydrin-positive material using extracts in cold 80% ethanol. We used a technique described by Clark (1964) and are indebted to her for earlier personal communication of the method. The procedure was calibrated periodically using glycine standards, and such standards were determined routinely with unknown samples. The ninhydrin-positive material

in the extracts is treated as free amino acid and concentrations expressed as millimoles amino acid per kg. body water.

Uptake of amino acids was measured by supplying randomly labelled glycine- C^{14} or *l*-isomers of the other amino acids employed. Concentrations greater than 10^{-6} moles per liter were obtained by adding unlabelled amino acid. Determinations of radioactivity were made using a thin-window gas flow detector system. Animals were exposed to sea water solutions of labelled amino acids for a predetermined time. Initial and final radioactivity in the sea water was determined. Each individual was extracted for 24 hours in 2.0 ml. of 80% ethanol. Five-tenths-ml. samples of this extract were evaporated on planchets and counted. Each individual was then ground in 2.0 ml. of distilled water and 0.5-ml. samples of the brei evaporated on planchets. All data presented have been corrected for background and sample thickness.

Care was taken to insure that the data collected in one particular set of observations would be internally comparable by preparing labelled solutions from a single stock to the same final concentration. Thus no corrections for small differences in ambient radioactivity were required.

Descending paper chromatograms were prepared using *n*-butanol-acetic acid-water (120:30:50) followed by phenol-water (80% by weight) as described by Smith (1960). One-dimension descending chromatograms were also prepared using *n*-butanol-acetic acid-water. Autoradiographs were prepared by exposing Kodak No-Screen x-ray film to the chromatograms for a seven-day period.

RESULTS

When exposed to a solution of glycine- C^{14} , uptake of the radioactive label is rapid and approximately linear for at least 30 minutes. Under normal circumstances, the greater part of the radioactivity is in the alcohol-soluble fraction while only a small percentage of the total is found in the brei. At the end of a 30-minute exposure, the ratio of alcohol-soluble to alcohol-insoluble radioactivity is of the order of 30:1.

A one-dimensional chromatogram of the alcohol extract of *Ophiactis* shows several ninhydrin-positive spots. The most prominent have R_f values which agree with those of glycine, alanine, taurine, and threonine. In both one- and two-dimensional chromatograms, autoradiographs show radioactivity in the region identified as glycine. Hence, it appears that the great bulk of the radioactivity is still in the form in which it was supplied. This was also true of an alcohol extract prepared from animals which had been sacrificed 24 hours after a 30-minute exposure to labelled glycine.

A number of experiments were performed using a larger brittle star, *Ophionereis annulata*. The animals were induced to autotomize their arms, and uptake of glycine by the isolated arms was measured. With suitable corrections, it appears that this preparation is about as effective as the whole animal, at least for three or four hours. Consequently, it is likely that the gut is not involved in any extensive way in this uptake. This would agree with previous reports (Stephens, 1962, 1963, 1964) for other invertebrates, and very probably applies to *Ophiactis* as well.

Observations were undertaken to relate uptake of labelled glycine to the weight of the animals. When the log of uptake was plotted against the log of wet weight,

a regression line of slope 0.545 was calculated by the least squares method. Rather than correcting for weight based on the exponential relation this implies, weights of the animals employed for subsequent observations were kept as closely comparable as possible and within the range of 10 to 30 milligrams. Uptake is expressed as cpm./mg. for animals in this range.

Observations relating uptake to ambient concentration of amino acid were carried out using glycine, valine, alanine, and arginine. When the data were plotted as the reciprocal of concentration against the reciprocal of uptake, the straight line which was expected was not obtained. Uptake was systematically too high at high concentrations for all of the amino acids used. A more extended series of

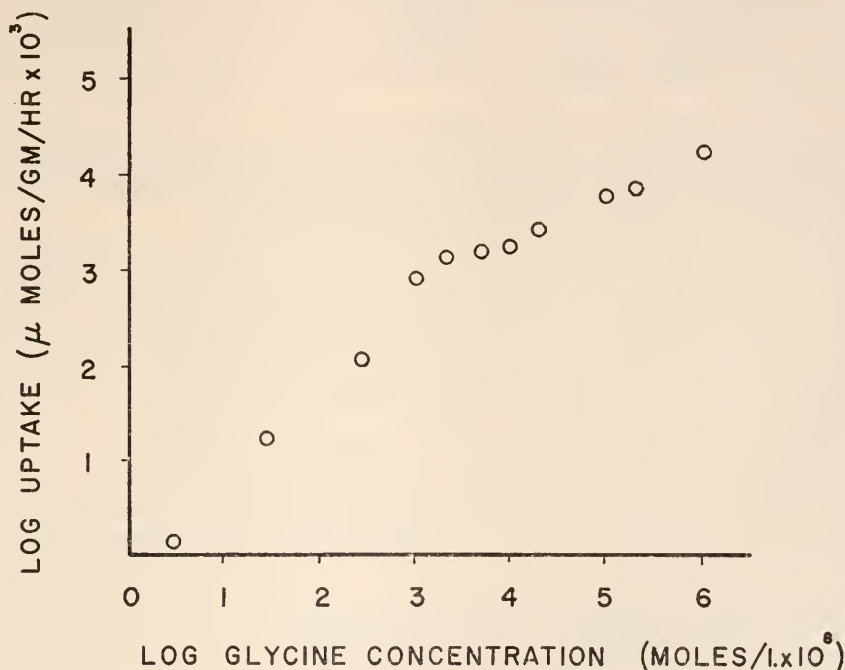


FIGURE 1. Rate of glycine uptake by *Ophiactis* as a function of ambient concentration of glycine. Each point represents the average value for ten or more individuals.

concentrations of glycine was used, covering the concentration range from 2.6×10^{-8} to 10^{-2} moles per liter. The data are presented in Figure 1. It will be noted that the accumulation system is not saturated at the highest concentration used. This differs from the relation reported for *Fungia*, *Clymenella*, *Nereis* sp., *Golfingia*, and a number of other invertebrates which show a definite maximum rate of accumulation (Stephens, 1962, 1963, 1964; Virkar, 1963, and unpublished observations). At the end of a 30-minute exposure to the lowest concentration employed ($2.6 \times 10^{-8} M$), about 64 times as much labelled carbon per kilogram of water was found in the alcohol-solution fraction of *Ophiactis* as was present in the ambient solution. The lower limit in concentration was imposed by the specific activity

TABLE I

Average radioactivity in the alcohol-soluble and alcohol-insoluble fraction of Ophiactis at various times after a 30-minute exposure to glycine-C¹⁴ (U.L.). The data are presented as cpm./0.5 ml. extract divided by weight in mg. Standard deviations are included; n is 10 for all groups

Time (hours)	(cpm./mg.) alcohol-soluble	(cpm./mg.) alcohol-insoluble
0	117 ± 32	2.7 ± 0.7
0.5	108 ± 24	3.6 ± 0.9
1	106 ± 24	4.7 ± 1.2
2	111 ± 28	5.4 ± 0.9
4	106 ± 24	9.7 ± 2.3
6	86 ± 25	11.5 ± 2.7
24	45 ± 7	24.2 ± 4.4

of the labelled glycine and does not reflect a limitation of the physiological system involved. As is the case in forms previously examined, this accumulation system is essentially one way; no significant exchange of labelled material for unlabelled amino acid in the ambient medium was obtained.

When animals were exposed to glycine-C¹⁴ for 30 minutes and then allowed to remain in sea water for various periods subsequent to this exposure, there was a gradual increase in radioactivity in the alcohol-insoluble fraction of the animal. Table I lists the alcohol-soluble and alcohol-insoluble radioactivity at various times after a 30-minute exposure to labelled glycine. It is apparent that total radioactivity decreases with time although the alcohol-insoluble fraction increases in absolute level and not merely as a ratio.

At least a portion of the radioactivity which is lost from the system represents C¹⁴-labelled carbon dioxide. Water in which brittle stars have been placed for 24 hours after an exposure to labelled glycine shows some radioactivity. This disappears on acidification and can be trapped on alkali in a Conway diffusion flask. Table II presents a balance sheet accounting for 92% of the radioactivity initially present in the system. A later experiment showed that CO₂ was lost to the atmosphere before acidifying. About three-quarters of the radioactive CO₂ was trapped on alkali before the sea water was acidified. The measurements presented in Table II are thus systematically low. The estimated correction for loss to the atmosphere (the parenthetical figures in the table) is probably too large since

TABLE II

Assimilation of glycine-C¹⁴ by Ophiactis during 24 hours following a 30-minute exposure. Radioactivity is expressed as counts per minute per milligram. The parenthetical figures include an estimate for C¹⁴O₂ lost to the atmosphere based on separate measurements; n = 10 for all groups

Time	Alcohol extract	Brei	Medium	Total
0	573 ± 41	10.4 ± 1.9	—	583
24 hrs.	360 ± 67	150 ± 27	27 (114)	537 (624)

the free surface was not comparable in the two situations, but some additional CO_2 was certainly evolved.

A series of observations was undertaken to explore the effect of various inhibitors on accumulation of glycine by *Ophiactis* and its assimilation into alcohol-insoluble compounds. In each case, the animals were kept in solutions containing the inhibitor for one hour prior to exposure to labelled glycine in the presence of the inhibitor. Inhibitor concentrations of 10^{-3} , 10^{-4} , and 10^{-5} M were employed (except for N_2 where the animals were kept in nitrogen-saturated sea water). The data are summarized in Table III.

It is apparent that both accumulation and assimilation can be inhibited by a variety of agents. There is no reason to think that any of the inhibitors used has a specific effect on the system mediating accumulation. Both processes are sensitive to all of the compounds used. Assimilation seems to be reduced more than uptake in almost all cases. The inhibition noted probably reflects no more than

TABLE III

Percentage of inhibition of accumulation and assimilation of glycine by the agents listed at the concentrations indicated. Glycine concentration was approximately 10^{-6} moles per liter. All differences are significant at the 1% level

Inhibitor \ Concentration	10^{-3}		10^{-4}		10^{-5}	
	Alcohol	Brei	Alcohol	Brei	Alcohol	Brei
KCN	33%	66%	—*	58%	—	39%
2,4-DNP	47%	83%	26%	—*	—*	—*
IAA	36%	74%	—	47%	—	45%
N_3^-	—	25%	—	—	—	—
$\text{N}_2^\ddagger$	43%	54%				

* Difference at the 5% level of significance.

‡ Nitrogen-saturated sea water.

the general dependence of the organism on oxidative metabolism. It may be noted that these animals do not survive for more than a few hours in nitrogen-saturated sea water. The responses of *Ophiactis* contrast with the insensitivity to various inhibitors reported for *Clymenella* and the latter's capacity to tolerate long periods without oxygen (Stephens, 1963).

The water content of animals acclimated to salinities between 100% sea water and 50% sea water was determined. Measurements were also made of the alcohol-soluble ninhydrin-positive material. These data are presented in Figure 2. The ninhydrin-positive material is treated as free amino acid. Ten amino acids were tentatively identified from chromatograms on the basis of their R_f values. In view of the reports of taurine in echinoderms (Kittredge *et al.*, 1962), the o-phthalaldehyde color reaction (Smith, 1960) was used to check for its presence in chromatograms. It was shown to be present by this criterion. However, the largest single constituent in the amino acid pool was glycine.

It is clear that decreasing salinity is correlated with a decrease in ninhydrin-positive material in alcohol extracts. Uptake of glycine and of valine was observed

at various salinities, and assimilation into alcohol-insoluble material also determined. The measurements of radioactivity were made on alcohol extracts and breis prepared after a 30-minute exposure to labelled material and a brief rinse in sea water. Figure 3 summarizes the results for a typical set of observations employing glycine. The initial increase in the rate of accumulation as slightly lower salinities are encountered by the organism is a constant feature of our observations of this kind. We also consistently find an increase in the percentage of labelled material

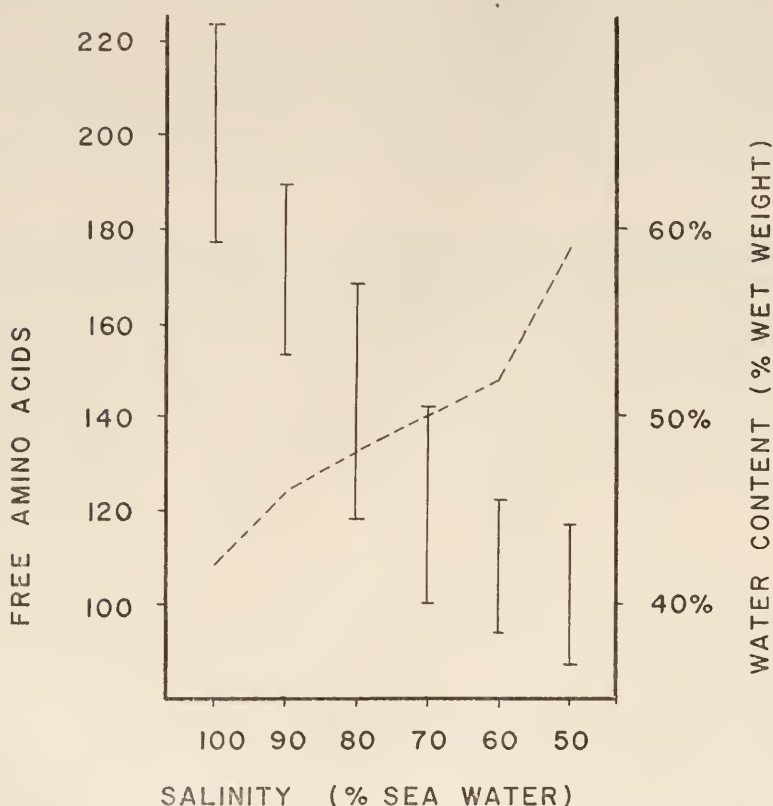


FIGURE 2. Total free amino acids (expressed as millimoles per kilogram body water) and water content of *Ophiactis* as a function of external salinity. Vertical bars represent standard deviations for amino acid values, dashed line the water content; n is 10 for amino acid determinations, 5 for water content.

accumulated which is assimilated into an alcohol-insoluble form. Observations with valine are similar. However, although the ratio of C^{14} assimilated increases with decreasing salinity, the absolute rate at which C^{14} appears in breis prepared after exposure at low salinities (50%, 60%) may decline. In any case, the specific stimulation of the assimilation of labelled carbon into the alcohol-insoluble fraction, concomitant with decreased uptake, contrasts with the effect of all inhibitors studied.

A set of observations was carried out to study the time course of the change in level of the free amino acid pool as well as the time course of the changes in accumulation and assimilation. Animals were transferred from 100% sea water to 60% sea water. Groups of ten animals were exposed to glycine- C^{14} and sacrificed at intervals for ten days following the transfer. The radioactivity of the

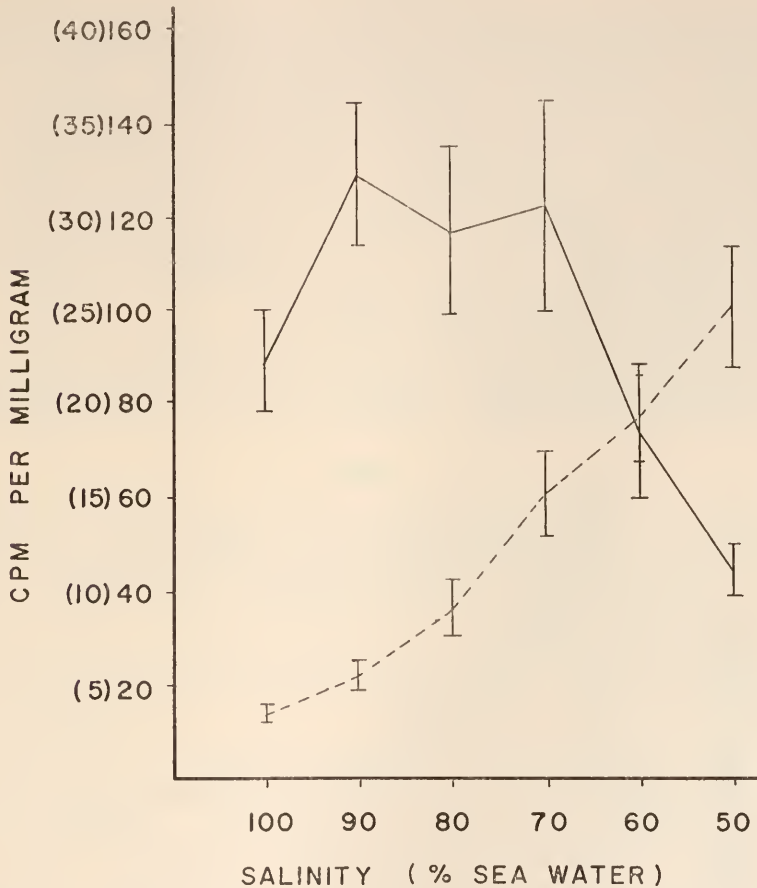


FIGURE 3. Radioactivity (expressed as counts per minute per milligram in 0.5 ml. extract or brei) recovered in the alcohol-soluble (solid curve) and alcohol-insoluble (dashed curve, parenthetical figures on ordinate) fractions of *Ophiactis* adapted to various salinities. The animals were exposed to glycine- C^{14} in their respective media for 30 minutes; n is 10 in all cases. Vertical bars represent standard deviations.

alcohol-soluble and the alcohol-insoluble fractions was determined for each animal. Measurements were made of the free amino acid pool in each group. A control group which was kept in 100% sea water under otherwise comparable conditions was sampled during the same period. Figure 4 presents the change in the free amino acid pool during the ten-day period for the two groups. Figure 5 presents

the percentage of the total radioactivity which was found in the alcohol-insoluble fraction of the animals. It will be noted that the response of the free amino acid pool is rather slow. This is also true with respect to the stimulation of incorporation of radioactivity into alcohol-insoluble compounds.

DISCUSSION

Like almost all other marine invertebrates which have been examined, *Ophiactis* is capable of removing amino acids from extremely dilute solution in the surrounding sea water. The failure to obtain a definite maximum velocity of uptake contrasts with results which have been reported previously. The continued increase in rate which was observed over an ambient concentration range covering six orders of magnitude suggests a diffusion process. However, we will note later that the "free" glycine pool exceeds even the highest of the external concentrations employed by a factor of ten. It should be reemphasized that there is an overall accumulation of amino acids, that amino acids do not freely exchange with the medium, and that the concentration of amino acids in the normal habitat of these organisms must be very low compared to the intracellular pool.

Little is known about the feeding habits of this particular animal. It is also difficult to defend any very specific comments about the possible contribution that might be made by dissolved organic compounds. The close association of *Ophiactis*

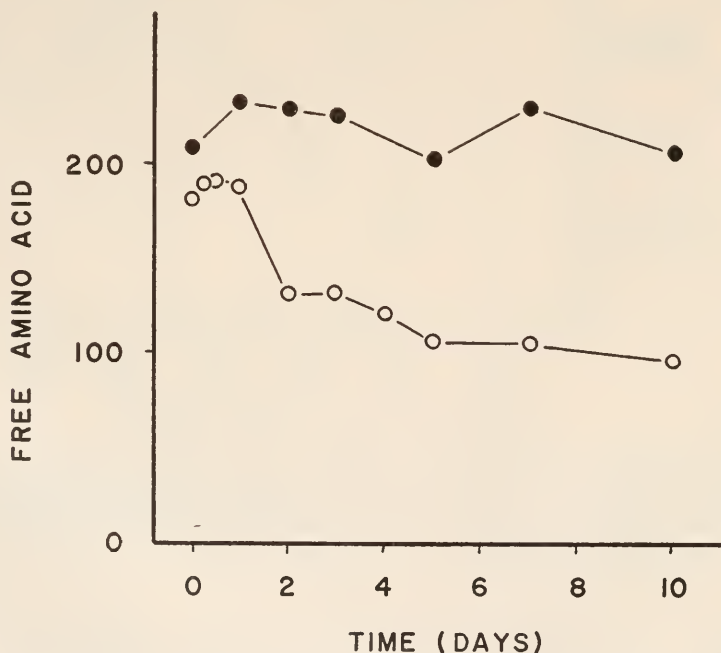


FIGURE 4. Time course of change in free amino acid concentration of *Ophiactis*. Concentration is expressed as millimoles per kilogram body water. Animals were transferred from 100% sea water to 60% sea water at zero time. Open circles, 60 % sea water; solid circles, controls in 100% sea water. Each point is average of ten animals.

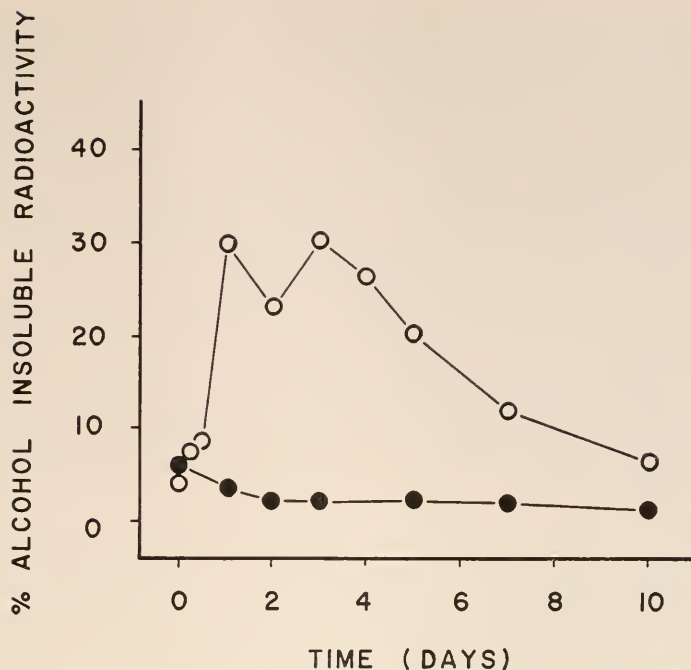


FIGURE 5. Radioactivity in the alcohol-insoluble fraction expressed as percentage of total radioactivity in *Ophiactis* exposed to glycine- C^{14} for 30 minutes at various periods of time following transfer to 60% sea water. In every case, ten animals each in 60% and 100% sea water were used. Open circles, 60% sea water; solid circles, 100% sea water.

with mussel beds and with sponges involves a microhabitat concerning which we have no chemical information whatever. Nonetheless, it is worth looking at the relation between the observed rate of amino acid accumulation and the metabolic needs of the animal.

Measurements of the oxygen consumption of animals in the size range of 10 to 30 milligrams wet weight, using a Gilson respirometer, gave approximately 0.147 ml. O_2 /gm./hr. This is roughly equivalent to 147 micrograms of glycine/gm./hr. Our measurements indicate that the animals can obtain 64 micrograms/gm./hr. at an ambient concentration of 10 micromoles glycine per liter (0.75 mg./l.). This is roughly the concentration of glycine measured in the interstitial water of mud flats (Stephens, 1963) and in inshore water samples (Belser, 1959, 1963). This represents 43% of the organic material necessary to support the observed oxygen consumption. Since we have no information concerning conditions in the immediate environment of these brittle stars, we can only note that very modest ambient concentrations would permit this pathway to make a significant contribution to the energy needs of the organism.

The production of $C^{14}O_2$ indicates that the amino acids entering the organism are available for oxidation. Assimilation of labelled carbon into the alcohol-insoluble fraction implies that this material contributes to synthesis pathways. A rough estimate concerning the relative magnitude of the contribution of the

amino acid pool to energy metabolism can be made. Although complete quantitative information concerning the individual amino acids in alcohol extracts is not available, rough estimates were made by comparing chromatograms with controlled chromatograms of known amounts of glycine and taurine. An estimate of 0.1 mole of glycine per kilogram of body water is reasonable for normal salinities. At least 5% of the labelled glycine which is accumulated appears as carbon dioxide in our observations (Table II). This implies that this percentage of the pool has been oxidized. This figure may be higher if one assumes that the labelled carbon which disappears from the system in the observations summarized in Table I represents oxidized material. The figure would then rise as high as 40%. The glycine pool represents about 7.5 mg./g. body water or about 3 mg./gm. wet weight at normal salinities. Hence 0.15 to 1.2 mg. of glycine enters oxidation pathways. The daily requirement on the basis of O_2 consumption is about 3.5 mg. Hence glycine might account for approximately 4% to 34% of this requirement. Although the estimate is rough, it probably brackets the typical contribution of the glycine pool to energy metabolism and indicates that it is ancillary and not the primary energy source.

We can estimate major outputs which influence the size of the glycine pool in these animals. Energy metabolism drains 0.15 to 1.2 mg. of glycine per gram wet weight from the pool per day. Assimilation into alcohol-insoluble compounds removes about 12% of the pool per day (Table I) or about 0.36 mg. per gram wet weight. We can ask whether rates of uptake measured in these animals could contribute to maintaining the pool in the face of these deficits. Again, we must simply assume some reasonable ambient concentration failing direct information. If we accept the figure of 10 micromoles per liter suggested above, the input to the pool amounts to slightly more than 1.5 mg. glycine per gram wet weight per day. The fact that this figure balances the losses indicated so closely is of course gratuitous. However, one may suggest that the uptake of amino acids from the ambient medium is potentially capable of maintaining the size of the free amino acid pool and supplying the known drains on that pool. This suggestion rests on the assumption that the modest amounts of free amino acid stipulated occur in the specialized habitat of this organism.

In common with many other marine invertebrates, *Ophiactis* responds to a reduction of salinity by a decrease in the pool of alcohol-soluble ninhydrin-positive materials. If allowance is made for the increased water content of the organisms, the pool decreases to about 83% of its normal size in 70% sea water. Further apparent decreases at 60% and 50% sea water are produced almost entirely by the increase in water content of the organisms. It should be noted that the present data are not comparable to those reported by Virkar (1963, 1965) in which a large initial response of the free amino acid pool was noted. It was possible to distinguish between intracellular fluid and coelomic fluid in the case of *Golfingia*. Since no such distinction was made in *Ophiactis*, one cannot directly assess the effectiveness of the reduction of the free amino acid pool in the latter as an osmoregulatory response. However, there is no inconsistency in the two reports.

Our observations indicate that the effect of a small decrease in salinity is an increase in both the rate of accumulation and the rate of assimilation of amino acids. As salinity is reduced further, the rate of accumulation drops but assimilation

increases in rate. At 50% sea water, the rate of uptake of glycine is about half its value at normal salinities but the rate of assimilation has increased by a factor of seven. If lowering the salinity merely interfered with the energy metabolism of the organisms, we would expect a decrease in the rate of assimilation as was the case with all the metabolic inhibitors employed.

There are two major ways in which one might account for the increase in rate of assimilation at reduced salinities. They are not mutually exclusive. As the size of the pool decreases, there would be an apparent increase in the rate of assimilation in our experiments because of the increased specific activity of labelled glycine in the pool. This may be an element in the observed response. It does not seem to be a sufficient explanation. The total decrease in alcohol-soluble amino acids amounts to about 50%; the apparent increase in rate of assimilation of glycine is about 700%. Although previous reports have indicated that particular amino acids such as glycine and alanine may be disproportionately involved in responses to salinity change, chromatography does not indicate any change in concentration of glycine of this magnitude in our animals. Hence, the specious increase in assimilation rate which would be produced by the decrease in size of the pool is probably not the most important effect reflected by our data.

The other possibility is that a decrease in salinity may produce an increase in the rate of incorporation of free amino acids into polypeptide and that this is the cause of the decrease in size of the pool. This seems reasonable. Incorporation of the free amino acids into an osmotically inactive pool would be the most economical way to reduce the pool. It is apparent from the magnitude of the increase in rate of assimilation that this could provide a rapid adjustment. At normal salinities about 3% to 5% of the pool is assimilated in a 30-minute period. In 50% sea water, about 36% of the pool is assimilated in the same time.

There is an apparent discrepancy between the time course of the change in the free amino acid concentration of *Ophiactis* (Fig. 4) and the time course of stimulation of incorporation of radioactivity into the alcohol-insoluble fraction of the animals (Fig. 5). The drop in the size of the pool follows this stimulation by as much as a day. A decrease in salinity must also produce an increase in the rate at which glycine is returned to the pool, so that it is more accurate to think of the salinity stress as stimulating both the exit from and the entry into the pool. The acclimation in turnover rate which is observed after several days in 60% sea water is accompanied by a maintained lower level of free amino acids. Presumably a new steady-state balance between inputs and outputs is reflected in the data. This slow change is reminiscent of the slow acclimation to increased salinity of the accumulation system in *Nereis limnicola* (Stephens, 1964). A detailed explanation of the decline in alcohol-insoluble radioactivity after prolonged exposure to reduced salinity awaits further work.

Since labelled carbon dioxide is produced, metabolism of the free amino acids cannot be limited to the shuttling back and forth from polypeptides to the pool which the autoradiographs might suggest. The specific activity of the glycine pool is very low despite considerable radioactivity in the alcohol-soluble fraction because of the remarkable concentration of the free amino acid pool. It is probably this which accounts for the failure to note intermediate compounds in oxidative pathways by autoradiography.

The pattern of regulation in *Ophiactis* which is suggested by these observations depends on regulation of the rate of synthesis and breakdown of polypeptide in response to a change in salinity. It is surprising to find that the initiation of these changes is as slow as it seems to be. The fact that 24 to 48 hours are required to produce a reduction in free amino acids in response to a sharp challenge suggests that the initial survival of the animal depends on its capacity to accommodate to drastic change. The great diversity of osmoregulatory mechanisms which have been described with regard to inorganic ion regulation make it very risky to attempt to generalize from the work we are reporting. We contemplate studies of other euryhaline animals to provide comparative data.

SUMMARY

1. The brittle star *Ophiactis arenosa* shows uptake of C^{14} -labelled glycine, valine, alanine and arginine from dilute solution. The process is linear with time for at least 30 minutes.

2. The bulk of the radioactivity accumulated during a 30-minute exposure to glycine- C^{14} remains in alcohol-soluble form. Autoradiography reveals the radioactivity to be associated with glycine.

3. If the animals are allowed to remain in sea water following such an exposure, there is a gradual assimilation of the label into alcohol-insoluble compounds. Some radioactivity appears as $C^{14}O_2$, implying oxidation of the amino acid.

4. A double-reciprocal plot of concentration against rate of uptake does not give a straight line. Even at ambient concentrations as high as 10^{-2} M, the accumulation system apparently is not saturated.

5. Common metabolic inhibitors decrease the rate of both accumulation and assimilation.

6. The free amino acid pool of *Ophiactis* in 100% sea water is of the order of 200 mM/kg. body water. In animals subjected to reduced salinities, there is a decrease in the size of the pool corresponding to the degree of dilution of the medium.

7. One- and two-dimensional chromatograms of alcohol extracts of the animals show several ninhydrin-positive spots, of which glycine, alanine, threonine, and taurine are most prominent.

8. When animals maintained at reduced salinities are exposed to labelled glycine or valine, the response to modest decrease in salinity is a stimulation of uptake. As salinity is decreased further, there is a decrease in the rate of accumulation. In all cases, however, there is a marked increase in the rate of assimilation of the accumulated material into alcohol-insoluble compounds.

9. The response to reduced salinity, with respect to both the size of the free amino acid pool and the incorporation of the label into alcohol-insoluble fraction, is slow, occurring over a period of several days.

10. The significance of the results is discussed in terms of the energy relations of the animals, and the functions of the free amino acid pool.

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THE EFFECT OF TEMPERATURE UPON THE GROWTH OF LABORATORY-HELD POSTLARVAL PENAEUS AZTECUS¹

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The general life-history of North American shrimp of the genus *Penaeus* has been known for some time (Weymouth, Lindner and Anderson, 1933; Pearson, 1939; Burkenroad, 1934). Adults of the white shrimp, *P. setiferus*, the most intensely studied species, spawn offshore. The young move to the estuaries and, after a period of rapid growth, return to the offshore spawning grounds. During the estuarine phase of the life cycle, the postlarvae, and later the juveniles, are exposed to wide variations of temperature and salinity. Although it has been suggested that the lower salinities of the estuaries are necessary to the growth and survival of these postlarval penaeids (Pearse and Gunter, 1957; Gunter, Christmas and Killebrew, 1964), recent laboratory studies indicate that salinity *per se* has little effect on growth of postlarval *P. aztecus* (Zein-Eldin, 1963).

Zein-Eldin and Aldrich (1965) suggested that temperature was of greater significance than salinity for growth and survival of *P. aztecus*. Their experiments were, however, conducted at only four temperatures: 11°, 18°, 25°, and 32° C. The resulting data indicated greater differences in growth rate between groups held one month at 11° and 18° C. or those at 18° and 25° C., than between groups held at 25° and 32° C. The greatest growth-differential per 7 degrees was between 18° and 25° C.

As a result of these experiments, we decided to make a more exhaustive study of the effects of temperature in the range (15°–35° C.) commonly encountered by the postlarvae.

METHODS

Postlarval *P. aztecus* were obtained from the surf zone of the Gulf of Mexico at the entrance to Galveston Bay and kept in the laboratory for 24 hours before introduction into experimental aquaria. Postlarvae were tested at five constant temperatures in each of two series. In Series 1, we tested at temperatures of 15° through 25° C. at intervals of 2.5°, and in Series 2, temperatures of 25° through 35° C. at the same intervals. Animals were obtained for Series 1 in April, 1964, from water of about 23.5° C., and for Series 2 in August, 1964, from water of about 29° C.

Groups of 20 postlarvae were placed in glass aquaria containing 4 liters of continuously aerated and filtered bay water (salinity approximately 25‰). Five such aquaria, prepared as described by Zein-Eldin (1963), containing a total of 100 animals, were held in darkness at each constant temperature in B.O.D.-type

¹ Contribution No. 216, Bureau of Commercial Fisheries Biological Laboratory, Galveston, Texas.

TABLE 1
Mean animal weight (mg.) and length (mm.) at each temperature level ($^{\circ}\text{C}.$)
Values are based on samples of 10, except where indicated. Figures in parentheses indicate one standard error

Temperature	Series 1									
	15 $^{\circ}$		17.5 $^{\circ}$		20 $^{\circ}$		22.5 $^{\circ}$		25 $^{\circ}$	
	Mean length (mm.)	Mean weight (mg.)	Mean length (mm.)	Mean weight (mg.)	Mean length (mm.)	Mean weight (mg.)	Mean length (mm.)	Mean weight (mg.)	Mean length (mm.)	Mean weight (mg.)
0	12.0 (0.3)	5.1 (0.2)	12.0 (0.2)	5.1 (0.2)	12.0 (0.2)	5.1 (0.2)	12.0 (0.2)	5.1 (0.2)	12.0 (0.2)	5.1 (0.2)
5	12.2 (0.3)	5.2 (0.4)	12.0 (0.2)	7.0 (0.4)	12.4 (0.3)	8.2 (0.3)	12.6 (0.3)	9.0 (0.7)	13.5 (0.3)	12.2 (0.7)
10	12.7 (0.2)	7.4 (0.5)	12.4 (0.3)	8.7 (0.7)	12.8 (0.4)	10.5 (1.2)	13.5 (0.3)	14.7 (1.2)	14.6 (0.8)	29.1 (4.1)
14	11.8 (0.3)	7.0 (0.8)	12.6 (0.3)	9.6 (0.7)	13.9 (0.5)	15.2 (1.4)	16.7 (0.6)	30.0 (1.2)	18.4 (1.0)	49.9 (9.6)
20	12.0 (0.2)	7.4 (0.4)	13.2 (0.5)	12.8 (1.5)	16.6 (0.6)	28.2 (3.0)	18.9 (0.6)	43.8 (3.8)	22.6 (2.0)	90.2 (18.3)
25	12.2 (0.2)	7.9 (0.5)	14.4 (0.2)	15.2 (0.8)	19.1 (0.9)	43.3 (6.1)	21.2 (0.8)	64.7 (7.5)	28.5 (0.9)	158.7 (13.6)
31 Sample of 10	12.7 (0.3)	9.6 (0.8)	15.0 (0.3)	17.6 (1.4)	20.8 (0.6)	50.9 (4.4)	24.3 (1.2)	97.2 (7.0)	34.0 (1.3)	264.3 (33.2)
All survivors	12.6 (0.2)	9.8 (0.5)	14.5 (0.2)	16.7 (0.8)	20.7 (0.3)	52.2 (2.9)	23.9 (0.6)	90.6 (2.9)	33.0 (0.8)	241.7 (18.4)
No. of survivors	30		37		44		41		29	
No. of artificial deaths	2		2		5		7		19	
No. removed for sampling	52		50		50		51		49	

Temperature	Series 2									
	25 $^{\circ}$		27.5 $^{\circ}$		30 $^{\circ}$		32.5 $^{\circ}$		35 $^{\circ}$	
	Mean length (mm.)	Mean weight (mg.)	Mean length (mm.)	Mean weight (mg.)	Mean length (mm.)	Mean weight (mg.)	Mean length (mm.)	Mean weight (mg.)	Mean length (mm.)	Mean weight (mg.)
0	9.4 (0.1)	3.6 (0.2)	9.4 (0.1)	3.6 (0.2)	9.4 (0.1)	3.6 (0.2)	9.4 (0.1)	3.6 (0.2)	9.4 (0.1)	3.6 (0.2)
5	10.4 (0.2)	5.4 (0.6)	10.9 (0.3)	6.7 (0.6)	11.6 (0.3)	8.7 (1.0)	11.4 (0.4)	8.5 (1.0)	10.6 (0.2)	7.0 (0.7)
11	13.4 (0.4)	16.3 (1.2)	15.9 (0.6)	29.1 (2.8)	17.0 (0.5)	35.5 (3.3)	16.4 (0.7)	33.4 (4.0)	10.9 (0.2)	7.4 (0.5)
15	16.0 (0.3)	33.0 (2.2)	20.5 (0.7)	57.5 (6.0)	21.5 (1.0)	72.2 (8.8)	19.6 (0.9)	55.5 (6.6)	11.2 (0.4)	8.7 (1.1)
20	21.0 (1.3)	65.5 (8.9)	21.8 (1.6)	76.8 (15.2)	25.0 (1.2)	108.4 (14.2)	25.3 (1.0)	113.6 (13.9)	—	—
25	24.7 (0.9)	101.9 (12.2)	25.1 (1.7)	124.6 (18.5)	31.0 (1.7)	224.6 (35.8)	29.0 (1.7)*	199.4 (36.7)	—	—
29 Sample of 10	27.2 (1.0)	140.4 (13.6)	31.2 (1.9)	230.7 (32.7)	32.2 (2.6)	272.9 (46.0)	34.0 (1.7)	299.6 (47.5)	—	—
All survivors	26.4 (0.4)	125.6 (6.2)	30.5 (1.2)	219.8 (20.4)	32.3 (1.8)	265.8 (32.1)	34.0 (1.7)	299.6 (47.5)	—	—
No. of survivors	37		30		15		10		0	
No. of artificial deaths	5		7		14		19		30	
No. removed for sampling	50		50		50		45		20	

* Sample of 5.

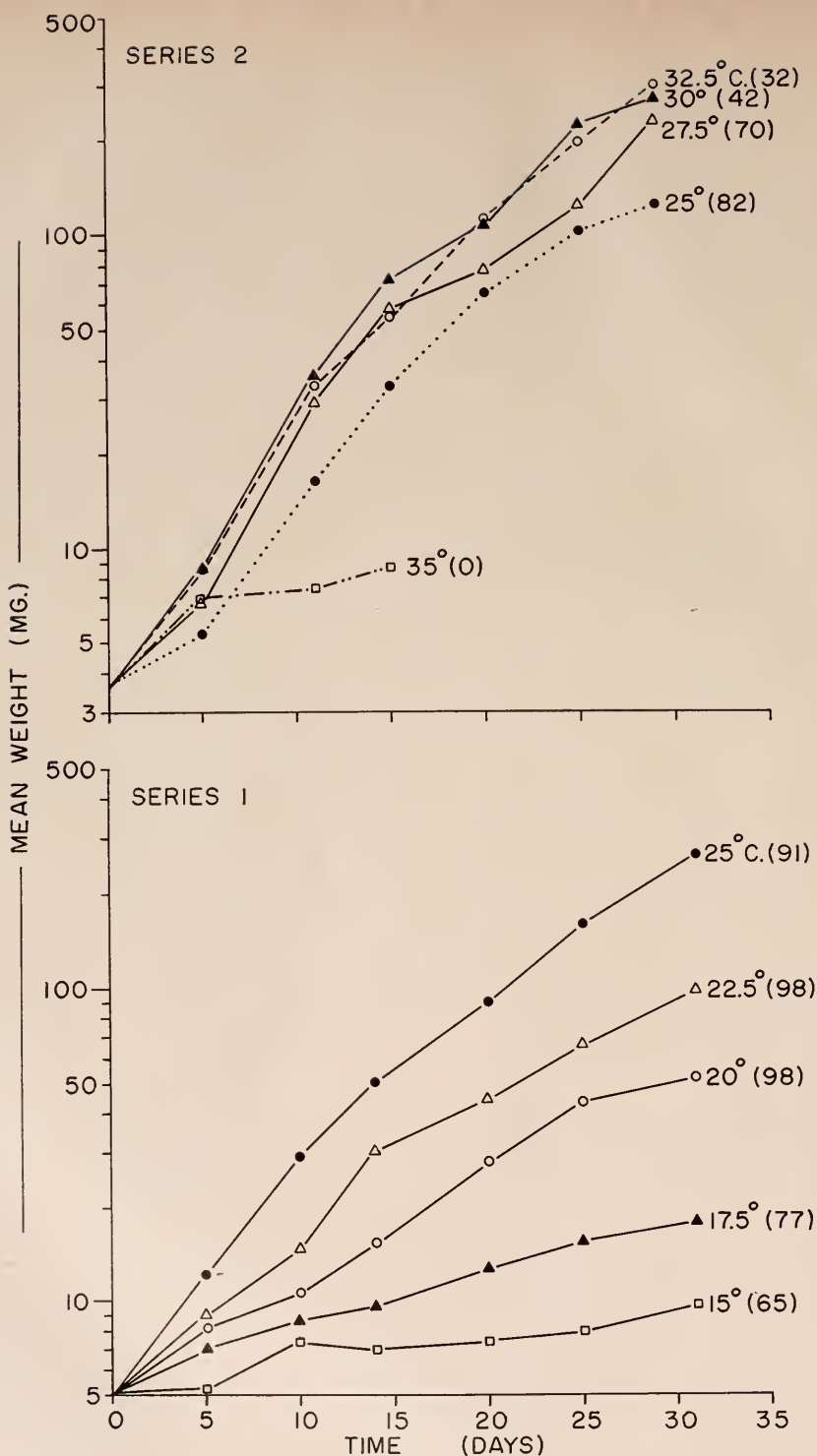


FIGURE 1. Mean increase in weight of *P. aztecus* postlarvae exposed to different temperatures ($^{\circ}\text{C}.$) for one month. Figures in parentheses indicate per cent survival.

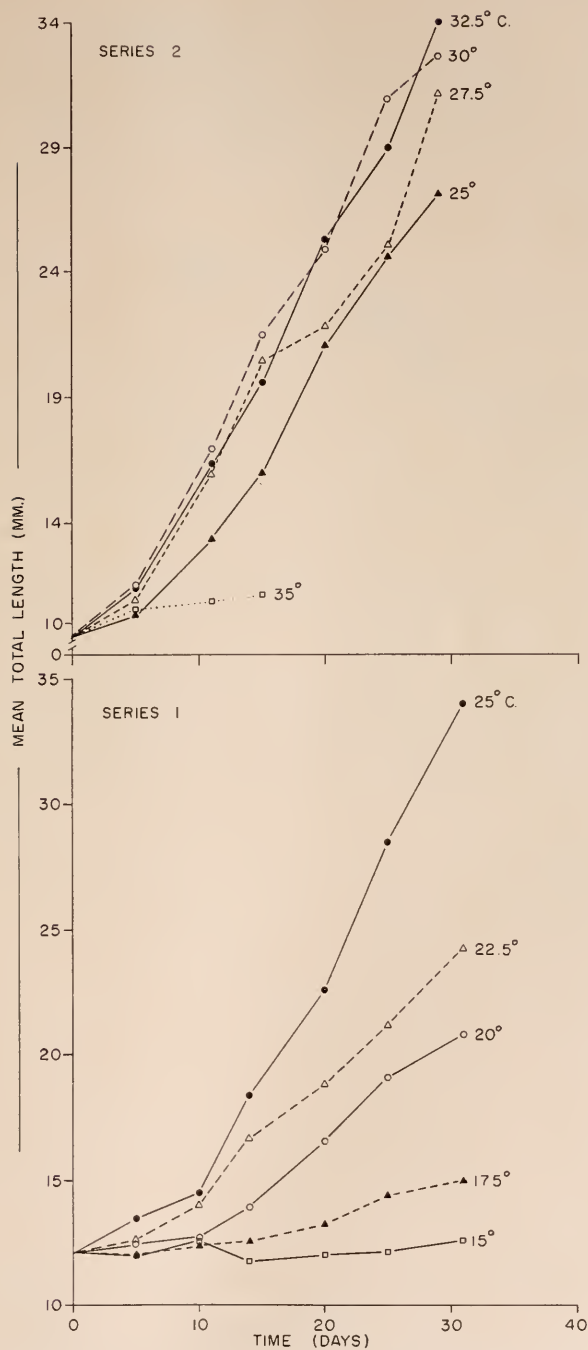


FIGURE 2. Mean increase in length of *P. aztecus* postlarvae exposed to different temperatures for one month.

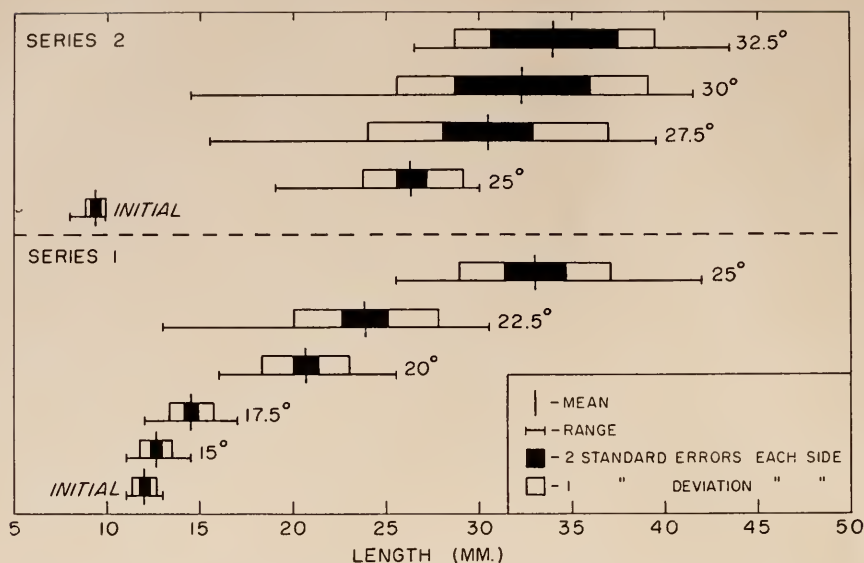


FIGURE 3. Length range (mm.) of postlarval *P. aztecus* surviving exposure to various temperatures for one month.

incubators. The incubators were adjusted over a period of 36 hours from room temperature (approximately 23° C.) to the final experimental temperature.

Experimental animals were fed sufficient live nauplii of brine shrimp (*Artemia*) to keep food constantly available during the month of the study. At each sampling

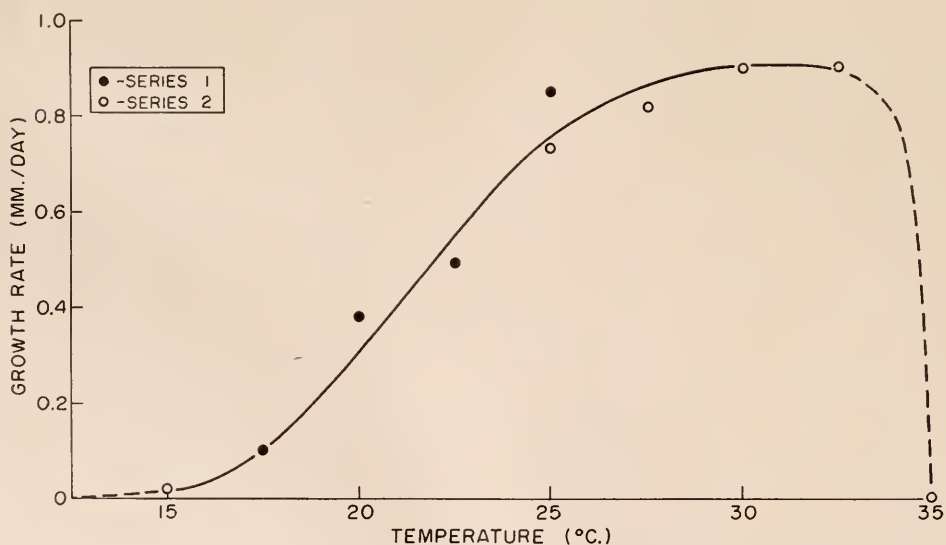


FIGURE 4. Growth rate (mm./day) of *P. aztecus* postlarvae held one month at different temperatures. Rate determined by inspection from straight-line portions of curves in Figure 2.

period (about 5-day intervals), two postlarvae were taken from each aquarium. The 10 animals from a given temperature constituted a sample. Postlarvae were weighed and measured individually as described by Zein-Eldin (1963). At the completion of the study, all remaining shrimp were weighed and measured individually to check the reliability of the sampling procedure (Table 1) and to determine the percentage survival at each temperature.

RESULTS

Growth

The final mean size of the postlarvae, whether derived from length or weight (Figs. 1 and 2, and Table 1), increased with temperature between 15° and 32.5° C. Growth rate, however, decreased markedly at 35° C., an effect evident in the samples taken on the 11th day. The greatest difference in growth between adjacent temperatures occurred between 17.5° and 20° C. and between 22.5° and 25° C. (Fig. 3). As a result, the increase in growth rate per unit of temperature was greatest in the temperature range 17.5° to 25° C. This differential effect of temperature is illustrated in Figure 4, an S-shaped curve with a maximum (at 30°

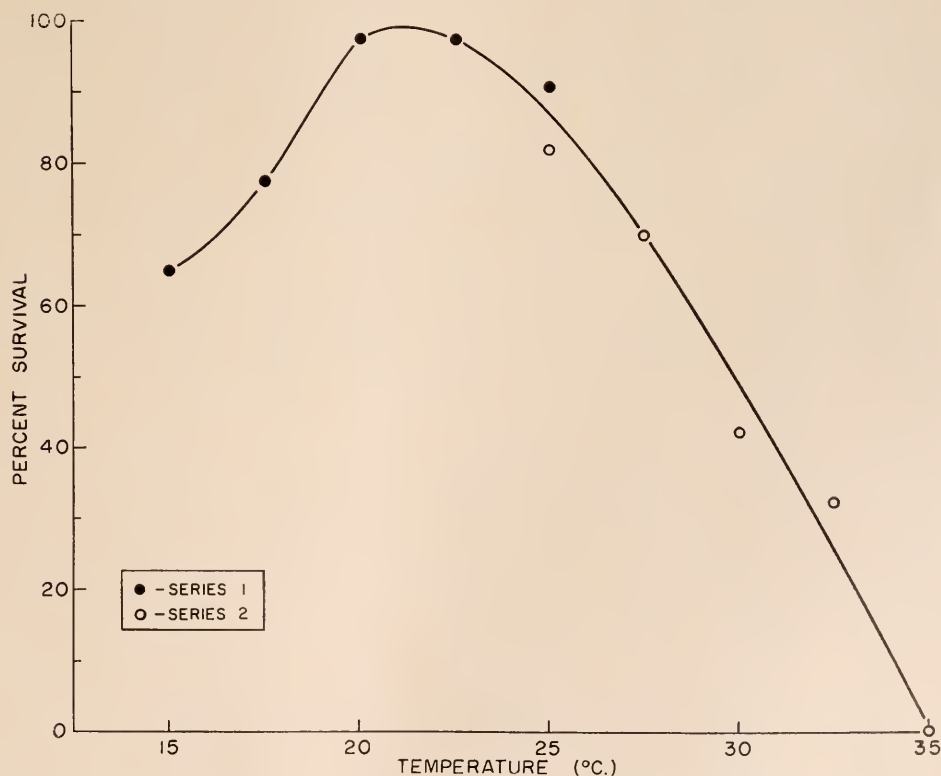


FIGURE 5. Per cent survival of *P. aztecus* postlarvae held at different temperatures for one month.

to 32.5° C.) typical of invertebrate growth responses to temperature (Needham, 1964, p. 416). In Series 2, final size did not differ significantly between groups exposed to temperatures of 27.5° to 32.5° C. (Fig. 3).

Survival

The relation of survival to temperature was somewhat different. The percentage survival increased with temperature between 15° and 20° C., remained above 90% at 22.5° and 25° C., but dropped at temperatures above 25° C. (Fig. 5). At 35° C., no animals remained after the 15th day. A similar decrease in survival at 32° C. as compared with 25° C. was noted in earlier experiments in our laboratory (Table II).

Gross production

Gross production, as estimated by comparing the total weight gains of the post-larvae surviving exposure to each temperature, was used to assess the combined

TABLE II
Comparison of growth of P. aztecus postlarvae from various experiments

Experiment no.	Date	Volume of experimental unit (liters)	No. of animals per experimental unit	Illumination*	Temperature (°C.)	Mean increase in length (mm.)	Duration of experiment (days)	Mean growth rate (mm./day)	Δ^t/Δ^{25**}	Survival (%)
GS-1†	4/62	45	100	+	24.0	19.0	28	0.68	1.00	36
GS-2†	8/62	45	100	+	26.0	17.4	29	0.60	1.00	100
GTS-1††	3-4/63	45	100	+	25.0	22.3	28	0.80	1.00	100
GT-F1	4/64	45	100	+	25.0	25.2	30	0.84	1.00	100
Series 1#	4/64	4	20	—	25.0	21.0	31	0.68	1.00	91
GTS-3	8/64	45	90	+	25.0	20.3	28	0.72	1.00	100
Series 2##	8/64	4	20	—	25.0	17.0	29	0.59	1.00	82
GTS-1††	3-4/63	45	100	+	32.0	32.0	28	1.14	1.38	58
GTS-2	8/63	45	40	+	32.0	30.4	29	1.05	—	34
GTS-3	8/64	45	90	+	32.0	27.2	28	0.97	1.35	100
Series 2##	8/64	4	20	—	32.5	24.6	29	0.85	1.46	32
GTS-1††	3-4/63	45	100	+	18.0	7.4	28	0.26	0.32	100
GTS-2	8/63	45	40	+	18.0	5.2	29	0.18	—	95
GT-F1	4/64	45	100	+	18.0	5.9	30	0.20	0.24	100
Series 1#	4/64	4	20	—	17.5	2.5	31	0.08	0.12	78
GTS-3	8/64	45	90	+	18.0	2.0	28	0.07	0.10	100
GTS-1††	3-4/63	45	100	+	11.0	0.5	28	0.02	0.025	92
GT-F1	4/64	45	100	+	11.0	0.0	30	0.00	0.00	26
GTS-3	8/64	45	90	+	11.0	1.1	28	0.04	0.05	5

* + indicates continuous fluorescent illumination; — continuous darkness.

** Δ^t = mean growth rate at temperature t ; Δ^{25} = mean growth rate at 25°.

† Data from Zein-Eldin, 1963.

†† Data from Zein-Eldin and Aldrich, 1965.

Animals from same population as those in GT-F1.

Animals from same population as those in GTS-3.

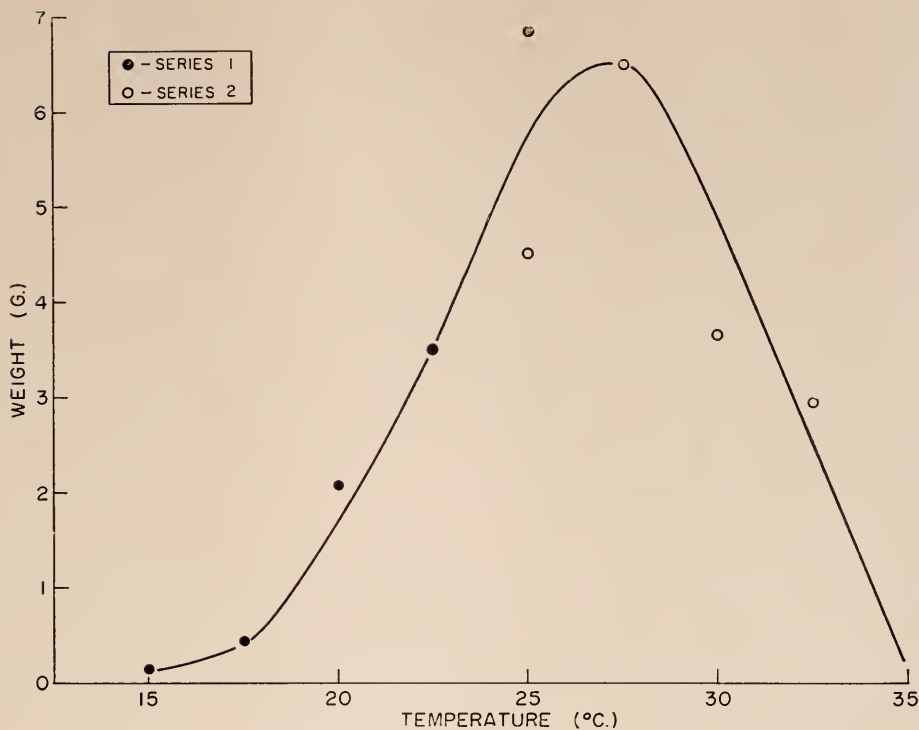


FIGURE 6. Increase in total weight of postlarval *P. aztecus* surviving different temperatures for one month.

effects of growth and mortality (Fig. 6). The total weight gain was maximal at temperatures of 25° and 27.5° C.; under laboratory conditions, the increased mortality at higher temperatures apparently has a greater effect on production than has the accelerated growth of survivors.

Food conversion

The efficiency of food conversion in these experiments was 10% to 15% lower than that determined in previous work in which large tanks and continuous light were used (Zein-Eldin and Aldrich, 1965). Efficiencies were approximately 30% at all temperatures except 15°, 17.5°, and 35° C. The efficiency declined to 15% at 17.5° C. and to 9% at 15° C.

Temperature relations

Growth rates at 25° C. were greater in Series 1 than in Series 2 (Figs. 1 and 2). The previous temperature history of the animals in the two series did not appear to explain the differences in either growth rate or survival. If past temperature history were a major factor in determining growth rate and survival, animals obtained in August would be expected to grow faster and survive better at high

temperatures (30° C. and greater) than those obtained in April when temperatures were considerably lower. Conversely, spring animals might be expected to grow and survive somewhat better at temperatures below 20° C. than animals obtained at higher August temperatures. To compare various wild populations exposed to the same conditions, we examined the growth data from several experiments (identified only by code numbers in Table II) performed in our laboratory during the past few years. Spring postlarvae did grow slightly more rapidly at 18° C., but animals collected in August neither grew more rapidly nor survived better at 32° to 32.5° C. than those collected in the spring (Table II).

To obtain further information concerning the effects of temperature on a given population, the mean increase in length of animals held at 25° C. in a given experiment was chosen as a standard, and the growth at other temperatures was compared with that at 25° C. (mean growth at temperature T divided by mean growth at 25° C.; Table II). The ratio of growth to that of the standard was nearly constant at 11° or 32° C., but was variable between 17.5° and 18° C. The variability at 17.5° to 18° C. may arise from the relatively great effect of temperature on the growth rate at this temperature range.

The somewhat reduced growth rates in these series were apparently caused by crowding. Higher growth rates were attained by animals from the same postlarval populations when reared in larger illuminated tanks (Experiments GT-F1 and GTS-3 of Table II). If the growth rates of animals from Experiment GT-F1 are compared with those of Series 1, or rates from GTS-3 with those of Series 2, rates in the two series are 0.8 of those in the larger tanks and, thus, correspondingly larger volumes of water. This reduced growth may have been the result of the smaller water volume per animal (crowding) rather than the lack of light, since subsequent experiments have indicated that effects of light are negligible.

It seems apparent that in laboratory studies, actual growth rates of the animals depend upon the particular natural population of postlarvae used. Comparisons of the relative effects of temperature are, however, valid within a given group of animals.

ECOLOGICAL IMPLICATIONS

A relation between temperature and growth rate of *P. aztecus* has also been suggested in reports on field studies by St. Amant, Corkum and Broom (1963), and Ringo (1965), who noted an apparent spurt of growth when the water temperature exceeds 20° C. Our results indicate that this pattern is a direct effect of temperature on the growth rate. The laboratory studies reported here also confirm the suggestion of Zein-Eldin and Aldrich (1965) that the influence of temperature on growth of postlarval brown shrimp is most marked in the 18° to 25° C. range.

Within the range of 15° to 20° C., small differences in temperature have a pronounced effect on the time needed for the completion of postlarval development in the laboratory (Fig. 7). The calculated time required for an average laboratory-held postlarva to increase from 12 to 25 mm. decreases from 260 days at 15° to 108 days at 17.5°, and to 36 days at 20° C. Temperatures greater than 20° C. bring about relatively minor decreases in the time required to complete postlarval development. That more rapid growth may occur in nature, where fluctuations

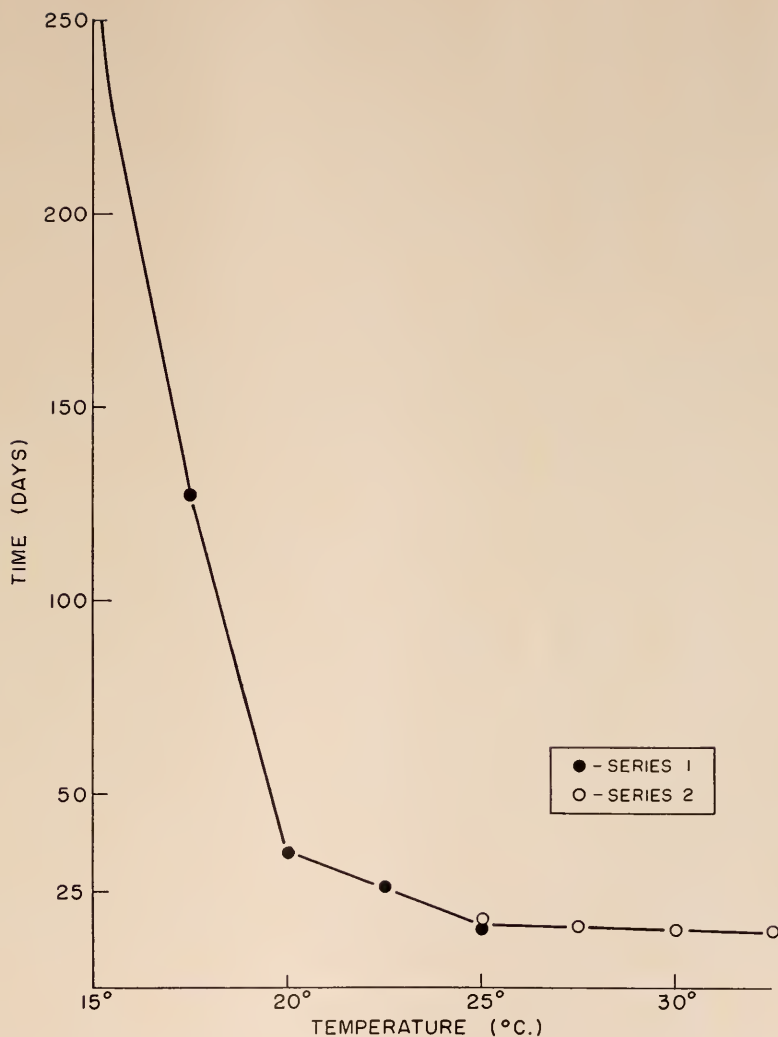


FIGURE 7. Number of days required for a 12-mm. postlarva of *P. aztecus* to grow to 25 mm. at different temperatures (based on slopes in Figure 4).

in temperature are the rule, must be re-emphasized. It is probable, however, that the growth rate below 20° C. is too slow to be readily observable in successive field samples.

Further observations are required to determine the degree to which other natural factors, such as food and light, influence the growth of postlarval *P. aztecus*, as well as that of *P. setiferus*.

SUMMARY

1. The growth of postlarval brown shrimp, *Penaeus aztecus*, was studied in the laboratory at constant temperatures of 15° through 35° C.

2. Growth increased with temperature up to 32.5° C. Maximal increases of growth rate per unit of temperature were observed in the temperature range of 17.5° to 25° C.

3. Survival for one month was markedly decreased at 32.5° C., and no animals survived at 35°.

4. The results suggest that in the laboratory gross production is optimal at temperatures of 22.5° to 30° C.

5. Non-lethal temperatures can have a strong effect on the time required to complete postlarval development.

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MECHANICAL FORCES AS A CAUSE OF CELLULAR DAMAGE BY FREEZING AND THAWING

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During microscopic examination of mammalian red blood cells exposed to freezing and thawing Smith, Polge and Smiles (1951) did not observe any intracellular ice crystals between the time the ice formed in the surrounding Ringer's solution and the time the cells became hemolyzed. When amoebae in pond water were subjected to a similar procedure, intracellular ice formation occurred. The presence of internal ice crystals in the amoebae was always associated with rupture of cell membranes and the amoebae in question never revived. Their experiments reveal that there may be differences among animal cells in their responses to freezing at a fixed temperature. The role played by ice formation *per se* is uncertain.

There are several potential factors involved with freezing injury to animal cells. On the one side we have the mechanical forces attendant upon ice crystal formation and on the other the physical and chemical changes such as hypertonicity or shift in pH associated with withdrawal of water from solution. The mechanical factors involved must be expected to be less dependent on the length of time the cells are exposed to freezing than are the physical and chemical ones. Intracellular ice formation is most likely to occur in supercooled cells (Mazur, 1963). Cells will become supercooled at freezing rates which are so high that intracellular water can not pass through the cell membrane rapidly enough to keep the concentration of solutes inside the cell in equilibrium with that of its surroundings. Assuming similar qualities for their cell membranes a suspension of small cells would therefore have to be exposed to more rapid freezing to show intracellular ice formation than would a suspension of large cells. The red blood cells of the congo eel have a diameter which is 10 times the diameter of the mammalian red blood cells studied by Smith, Polge and Smiles (1951). Visual observation during freezing at the freezing rate required to produce intracellular crystallization in mammalian red blood cells may be impossible, while the red blood cells of the congo eel are likely to become frozen internally at freezing velocities which permit observations of the freezing process. For this reason red blood cells from congo eels were utilized in an attempt to disclose whether freezing injury due to mechanical forces could be observed separate from eventual damage resulting from chemical changes. The cells could be studied during freezing and thawing over periods short enough to avoid hemolysis produced by changes other than the mechanical ones associated with ice crystal formation.

METHODS

Fresh samples of venous blood from the congo eel (*Amphiuma tridactylum*) with nucleated red blood cells of a diameter in the range of 70–90 μ were diluted

with Ringer's solution and a drop spread on a 1-mm.-thick slide of the acrylic resin "Perspex" and covered with a 0.2-mm. coverslip. A small piece of solid carbon dioxide in tinfoil was then placed at the edge of the coverglass, and the freezing of the diluted blood was watched through a Leitz Ortholux microscope with Leica camera for photomicrography. Magnifications between $40\times$ and $200\times$ were used. A thin layer of glycerol on the coverglass facilitated observation by avoiding condensation of water.

RESULTS

With the piece of solid carbon dioxide in place, ice formation in the diluted blood began immediately. The ice crystal front grew into the preparation and



FIGURE 1. The ice front is advancing through diluted blood from the congo eel. Shrinkage of the red blood cells starts as soon as they are reached by the spear-shaped ice crystals. The volume occupied by ice compared to the volume of the fluid space among the ice crystals indicates the portion of water withdrawn from solution. Magnification $100\times$.

water was withdrawn from solution. Shrinkage of the red blood cells started when they were reached by the ice front (Fig. 1) and continued concurrent with the decrease of fluid space among the ice crystals. At first ice crystals appeared exclusively in the suspending medium. Behind the ice front, however, ice crystals formed suddenly within one after the other of the shrunken cells. The crystals were so small that the light was scattered and the cell interior became opaque and appeared quite black (Fig. 2, A). When a definite extent of extracellular ice formation and thereby a certain degree of cooling was reached, the phenomenon would occur at various distances behind the ice front. The spontaneous intracellular freezing took place only as long as the ice front was advancing. The opaque

area sometimes appeared to occupy the whole cell interior, but in most experiments only the nuclear area and its immediate surroundings became opaque. If the piece of solid carbon dioxide at the edge of the coverglass was removed when a few of the red blood cells were frozen internally, the ice front receded (Fig. 2, B). With the exception of the cells with intracellular ice crystals the shrunken cells regained their shape, but some of them showed a ragged cell surface (Fig. 2, C). When

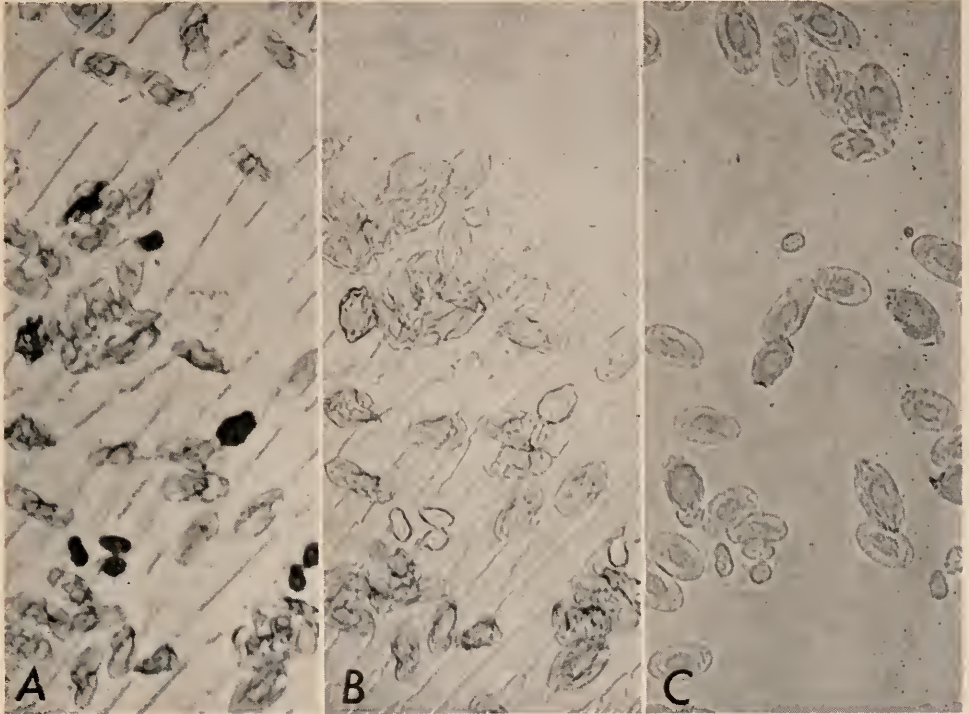


FIGURE 2. Diluted blood from the congo eel observed in three successive situations, A, B, and C, all observations made on the same field. A: The whole field is frozen and in a few of the cells intracellular ice formation is visibly manifest from the black appearance of their nuclear area. B: The ice front is receding. Dehydrated cells without intracellular ice crystals start regaining their normal shape. The opacity of the nuclear area of cells with intracellular ice crystals disappears. C: Thawing is complete. Some cells which have been dehydrated show a ragged cell surface, but only the cells with intracellular ice crystal formation are hemolyzed. The nuclei from the hemolyzed cells are left intact. Due to movements of fluid during thawing many of the cells have changed position, but five cells with intracellular ice crystals can be observed (as groups of three and two cells, respectively) in the lower part of the field (A). These cells are easy to trace through B and C. Magnification $100\times$.

the ice crystals of the internally frozen cells melted, the cells did not imbibe water to return to normal size. Their hemoglobin simply spread out into the suspending medium and apparently intact nuclei were left at the former sites of the whole cells (Fig. 2, C).

The ice front advanced more rapidly the nearer to the piece of solid carbon dioxide the field under observation was chosen. This was reflected in the sequence

of events as observed through the microscope. The description above and both figures refer to a field some distance away from the piece of solid carbon dioxide. Here the individual cells were easy to follow both during freezing and thawing. Further away the ice front advanced very slowly, and the cells seemed to become completely dehydrated without any signs of intracellular ice formation. Close to the edge where the freezing was initiated intracellular ice formation occurred almost immediately in all cells and dehydration could hardly be observed. Estimation of freezing temperatures from the amount of fluid space between extracellular ice crystals indicated that the slowly frozen cells would tolerate short-time freezing to lower temperature than the temperature at which all cells were destroyed at high freezing velocities.

By adjusting the size of the piece of solid carbon dioxide a frozen area which never reached the opposite edge of the coverglass was obtained. Different fields within this frozen area were kept under observation for 2–3 minutes with the ice front stagnant. The cells nearest to the edge where freezing started were all frozen internally. Somewhat further away some cells were frozen internally and some were dehydrated but unfrozen. Towards the ice front only dehydrated cells were found. Upon thawing all cells in the previously frozen area were hemolyzed except for a few ones close to the ice front.

DISCUSSION

The observations made during the present investigation show that two potential factors in cellular injury from freezing and thawing can be studied as separate processes by choosing appropriate experimental material and freezing rates. As an advocate for the great role of physical and chemical factors in freezing injury to animal cells, Lovelock (1953, 1957) presented experimental support for a theory involving a mechanism for cellular damage by freezing which would act without intracellular formation of ice. He showed that phospholipids and cholesterol were lost from the membranes of red blood cells suspended in solutions of sodium chloride, and that this loss was augmented with increasing concentration. The results were identical whether this increased concentration was brought about by freezing or by the addition of sodium chloride to the initial solution. The red blood cells in this way became more permeable to cations, and with an excess of cations the cells would slowly swell and hemolyze. Eventually they would be rapidly entered by water molecules and immediately hemolyzed when returned to physiological saline during thawing. The slowly frozen red blood cells from the congo eel which were dehydrated without any signs of intracellular ice formation and yet became hemolyzed if kept in this state for a few minutes before thawing apparently were destroyed according to this theory.

Sloviter (1962) emphasizes the possible damaging effect of mechanical forces attendant upon the formation of ice. He found that the extent of hemolysis after freezing and thawing of mammalian red blood cells suspended in non-electrolytes in the presence of different concentrations of sodium chloride did not increase with an increase in ionic strength of the surrounding medium of the cells. The destruction of red blood cells by sudden intracellular ice formation, as shown in Figure 2 in the present paper, demonstrates that such a mechanism for cellular

injury may occur. Although this process as studied in the present investigation seemed to be closely associated with the formation of ice crystals inside the cells, a weakening of the cell membranes, due to increased concentration of salts, may of course precede and promote the intracellular freezing. External ice crystals may also tear or penetrate the cell membrane and induce ice formation in supercooled cells.

The ice formed inside the red blood cells of the congo eel was not observed as individual ice crystals, but as a result of a scattering of the light when the crystals occurred in great number. The opaque area in most experiments covered only a field somewhat greater than the cell's nucleus, corresponding to the thickest part of the cell. Supercooling followed by rapid intracellular crystallization would be expected to promote the formation of ice throughout the cell. The failure to observe individual ice crystals in the thin portions of the cell may perhaps be ascribed to the low magnification used in this study. That supercooling is necessary for intracellular ice formation is indicated by the fact that the process was always associated with an advancing ice front. When the ice front is stagnant the individual cells are exposed to a constant temperature and apart from an eventual recrystallization with changes in ice crystal size, no alterations in the suspending medium will take place. The concentrations of solutes inside the cells will be in equilibrium with those of the outside. Very slow freezing will also allow equilibrium to be maintained and supercooling will be minimal.

The process of sudden intracellular ice formation with the appearance of cell opacity during freezing was described by Smith, Polge and Smiles (1951) to occur at about -8°C . for the amoeba. After thawing of the amoeba, its cell membrane was ruptured and cytoplasmic granules drifted out into the medium. Smith and Smiles (1953) found the same phenomenon to take place between -6° and -12°C . in preparations of tissues from guinea pig testis. The internally frozen cells from guinea pig testis were also disintegrated after thawing. This shows that the response of the red blood cells of the congo eel to freezing and thawing is related to the response of other cell types investigated, but intracellular ice crystal formation seems not to be inevitably lethal. Salt (1959) has demonstrated that the large cells in the fat body of the goldenrod gall fly, *Eurosta solidaginis*, can survive freezing even if intracellular ice crystals have been present.

Considering the frozen area where the ice front was kept stagnant for some minutes as described in the present paper, it appears that several ways of damage are demonstrated. Near the piece of solid carbon dioxide rapid freezing with intracellular ice formation, which destroyed all cells, occurred. A short distance behind the ice front the exposure to increased salt concentration during freezing probably was the only cause of injury. At an intermediate distance some cells seemed to be destroyed by one of these processes and some by the other one. The few cells quite close to the ice front which were found to recover after thawing show that freezing at low velocity to temperatures just below zero is not fatal provided the exposure is of short duration. This may correspond to the finding of Lovelock (1953) that the critical temperature range for red blood cells starts at -3°C . Once ice is formed in the preparation, the temperature at the ice front will be equal to the freezing point of the solution, and a temperature of -3°C . or lower must therefore be sought a short distance behind the ice front and

towards the piece of solid carbon dioxide. Judged from the present investigation it appears that, dependent on cell type and cooling rate, mechanical forces may be the predominant factor in cellular damage from freezing and thawing under some circumstances as may physical and chemical changes under others.

To avoid intracellular ice formation it is necessary to cool the cells at a slow rate. That rapid cooling promotes intracellular freezing is also shown for sea urchin eggs (Asahina, 1961) and for yeast cells (Nei, 1960; Mazur, 1961). However, during slow cooling the time of exposure of the cells to increased salt concentrations is prolonged as long as the temperature is above the eutectic points of these solutes. To obtain minimum damage two requirements therefore are to be met with. The cells must be cooled slowly enough to prevent intracellular ice formation and rapidly enough to minimize the damaging effects of exposure to increased salt concentrations. It is interesting, then, to note that for mammalian red blood cells such an optimum cooling velocity for minimum hemolysis has been demonstrated (Gehenio and Luyet, 1958).

I am grateful to Dr. Joseph Engelberg for careful reading of the manuscript. The investigation was supported by a research training award from the Norwegian Research Council for Science and the Humanities.

SUMMARY

1. The present investigation was initiated in an attempt to dissociate two potential factors in cellular injury from freezing and thawing: damage due to mechanical forces attendant upon ice crystal formation and damage due to physical and chemical changes associated with withdrawal of water.

2. Red blood cells in diluted blood from the congo eel were covered and subjected to microscopic examination during freezing induced by a piece of solid carbon dioxide placed at the edge of the coverglass. Ice crystals grew into the preparation, first rapidly and then more slowly the further from the piece of solid carbon dioxide they advanced. The sequence of events as observed through the microscope was different for different freezing velocities.

3. Rapid freezing caused intracellular ice formation and this internal freezing was always associated with hemolysis even if followed by immediate thawing. At slow freezing the cells became dehydrated without any signs of intracellular ice formation. Such cells would recover if thawing occurred within a few seconds, but they were all hemolyzed after prolonged exposure.

4. It is concluded that, dependent on the freezing rate, either mechanical forces or physical and chemical factors may be the main cause of cellular damage from freezing, the mechanical forces being predominant at rapid freezing.

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INFLUENCE OF INDIVIDUAL AMINO ACIDS ON UPTAKE AND INCORPORATION OF VALINE, GLUTAMIC ACID AND ARGININE BY UNFERTILIZED AND FERTILIZED SEA URCHIN EGGS¹

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In the course of investigations (*cf.* Tyler, 1965a) into the mechanism of the initiation of protein synthesis by sea urchin eggs, some variable results were obtained in tests with dactinomycin (actinomycin D). This inhibitor of DNA-primed RNA synthesis stimulated incorporation of labeled valine into protein in four experiments with suspensions of eggs that contained many oocytes but failed to do so in several subsequent tests. The nutritional status of the animals, and consequently of the eggs, was considered as one possible source of this variation. Tests were therefore made of the effects of glucose, which experiments by Honig and Rabinovitz (1965) had shown could prevent or relieve dactinomycin-induced inhibition of protein synthesis in sarcoma-37 cells. However, glucose did not enable dactinomycin to enhance incorporation of amino acid into protein by sea urchin eggs. Tests were then made with mixtures of amino acids. Again no stimulation was obtained with dactinomycin on the incorporation of a labeled amino acid. In these tests another phenomenon appeared, namely, a marked inhibition by the amino acid mixture on the incorporation of the labeled amino acid. The experiments on the oocytes, including the erratic dactinomycin effect, will be reported elsewhere (Piatigorsky, Ozaki and Tyler, 1966), while the present account will deal mainly with exploration of the competition among amino acids.

That the rate of uptake of one amino acid may be inhibited by the presence of others has been shown in many experiments with intact cells of various organisms (see Wilbrandt and Rosenberg, 1961; Christensen, 1962, 1964; Johnstone and Scholefeld, 1965, for review). In general, the inhibition is found to occur between members of the same general class of amino acid and is interpreted as being due to a competition for transport across the cell surface.

Since sea urchin eggs are the subject of increasing numbers of investigations of amino acid incorporation into protein by the intact cells, it seemed to us desirable to determine whether or not such competition at the cell surface occurs with this material, too, and if so, to examine the interrelationships among the amino acids. While this work was in progress a preprint was received of an article by Mitchison and Cummins (1966) concerning the uptake of labeled valine and cytidine by sea

¹ Supported by grants from the National Institutes of Health (GM 12777 and 2G-86) and the National Science Foundation (GB-28).

² Damon Runyon Cancer Research Fellow.

³ We wish to acknowledge the effective technical assistance of Peter N. Redington, Edgar E. Vivanco and Jeffrey W. Greene.

urchin eggs at various stages of development. In tests with eggs at one hour after fertilization they report a marked inhibition of the uptake of C^{14} -valine by each of five neutral amino acids (L-leucine, DL-isoleucine, DL-alanine, DL-phenylalanine, DL-threonine) and slight inhibition by one basic amino acid (DL-lysine).

Our experiments show that these findings hold also for unfertilized eggs and provide further evidence, from measurements of both accumulation and subsequent incorporation of amino acid into protein, supporting that of Mitchison and Cummins that the inhibition operates as a competition for entrance into the cell. We have extended the measurements to include all twenty of the "coded" amino acids tested, with both fertilized and unfertilized eggs, for ability to inhibit both uptake and incorporation into protein of a neutral (valine), acidic (glutamic acid) and basic (arginine) amino acid. In the present article the amino acids that are termed basic are histidine, arginine and lysine. The acidic group includes aspartic acid, glutamic acid and their derivatives asparagine and glutamine. The remaining thirteen of the "coded" amino acids are placed in the neutral group.

MATERIALS AND METHODS

Eggs were obtained from the sea urchin *Lytechinus pictus* by the method of KCl-injection, the suspension temporarily acidified to pH 5 to remove the gelatinous coat, and an aliquot removed for counting (Tyler and Tyler, 1966). For the tests of uptake and incorporation the eggs were incubated with the C^{14} -labeled amino acid and the C^{12} -amino acid being explored, in a total volume of 0.25 ml. of artificial sea water, at pH 8.0, in polystyrene test tubes, for the specified time and at 20° C. At the end of the incubation period a large excess (1 ml. of an ice-cold 0.1 M solution) of the C^{12} -amino acid, corresponding to the C^{14} -amino acid, was added as quencher. For the measurements of uptake the eggs were thoroughly washed with ice-cold artificial sea water and transferred with distilled water to filter papers which were rapidly dried and placed directly in the scintillation fluid⁴ in which radioactivity was determined (Tri-Carb spectrometer) with about 50% efficiency. For the measurements of incorporation the same filter papers were rehydrated by transfer through absolute alcohol, 95% alcohol and 5% trichloroacetic acid (TCA). They were then processed, as usual (Tyler, 1966), with hot TCA, the alcohols, and ether, and transferred to the vials of scintillation fluid for determination of incorporation of the labeled amino acid into protein.

RESULTS

1. Inhibition of uptake of C^{14} -valine by an amino acid mixture

As indicated above the initial experiments on the effect of additional amino acids on the incorporation of labeled valine were done in connection with tests of the action of dactinomycin. Table I gives the results of two experiments in which the incorporation of C^{14} -valine into protein was measured in the presence and absence of a mixture of amino acids (Borsook *et al.*, 1957) with and without dactinomycin. The inhibiting effect of the amino acid mixture is marked, regardless of whether or not dactinomycin is present. The latter had no significant effect on C^{14} -valine

⁴ 2.88 g. PPO (2,5-diphenyloxazole) and 0.34 g. dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) per liter of toluene.

TABLE I

Action of an L-amino acid mixture and of dactinomycin** on incorporation of C¹⁴-valine into protein by unfertilized eggs of L. pictus, incubated for 30 minutes at 20° C.*

Experiment	C ¹⁴ -valine (sp. act. 185 C./M) μc./ml.	Counts per minute per 10 ⁴ eggs			
		Without dactinomycin		With 0.015 mg./ml. dactinomycin	
		Without amino acid mixture	With amino acid mixture	Without amino acid mixture	With amino acid mixture
1	0.50	8327	216	10519	178
		9226	284	12112	173
2	0.42	1050	27	1078	14
		1701	24	1313	22

* Composition and final concentrations in mmoles/l.: Alanine, 0.33; arginine, 0.08; aspartic acid, 0.48; cysteine, 0.06; glutamine, 1.33; glycine, 0.89; histidine, 0.40; isoleucine, 0.05; leucine, 0.67; lysine, 0.30; methionine, 0.06; phenyl alanine, 0.26; proline, 0.23; serine, 0.28; threonine, 0.28; tryptophan, 0.05; tyrosine, 0.14.

** Gift of Merck, Sharp and Dohme, Rahway, N. J.; courtesy of Dr. H. B. Woodruff.

incorporation in these experiments. Differences between the two experiments in the absolute values for incorporation of C¹⁴-valine may reflect differences in size of the endogenous free valine pool in the eggs.

The presence of the added amino acids did not, then, enable the eggs to show a stimulated incorporation of C¹⁴-valine in response to dactinomycin, that had been previously noted with some batches of eggs of *Lytechinus* (see introduction).

2. Pretreatment with amino acids

In order to determine whether the inhibiting effect of the additional amino acids is on the accumulation of valine by the eggs or on its subsequent incorporation

TABLE II

Effect of pretreatment with an amino acid mixture (a.a. mix.) on the uptake of C¹⁴-valine by unfertilized eggs of L. pictus, incubated for 1 hour at 20° C.*

Pretreatment for 1 hour in:	Counts per minute per 10 ⁴ eggs			
	Total uptake		Incorporation into material precipitable by 5% trichloro-acetic acid	
	In presence of a.a. mix.	In absence of a.a. mix.	In presence of a.a. mix.	In absence of a.a. mix.
s.w.	80	23470	4	580
	100	27714	10	638
a.a. mix.	94	26442	8	1332
	132	27774	6	1338

* 0.53 μc./ml.; sp. act. 185 c./M.

into protein, tests were made on eggs that had been pretreated with the amino acid mixture and washed just before addition of the C^{14} -valine. Table II gives the results of one such experiment. The total uptake of C^{14} -valine, as well as the incorporation into protein, were determined. As the data show, preliminary exposure of the eggs to the mixture of amino acids has no effect on the subsequent uptake of the C^{14} -valine, either in the presence or in the absence of the C^{12} -amino acid mixture. But, uptake is almost completely suppressed by the amino acid mixture present during the period of incubation with the C^{14} -valine. The effect on uptake can, then, account for the inhibition of incorporation in the experiments shown in Table I.

The data of Table II also show inhibition of incorporation into protein in those eggs concurrently exposed to the amino acid mixture, regardless of prior exposure to the amino acid mixture. Furthermore there is an apparent increase in incorporation by those eggs exposed to the amino acid mixture before incubation with the C^{14} -valine alone. In three additional experiments an increase was obtained in one, while no appreciable difference was observed in the other two. At present, then, there is no consistent evidence that preincubation with other amino acids results in an increased incorporation of C^{14} -valine into protein.

Despite the washing following the pretreatment period the eggs probably retain most of the accumulated amino acids. This seems clear from experiments of other investigators (Nakano and Monroy, 1958; Mitchison and Cummins, 1966) and is indicated here by the large quantity of acid-soluble radioactivity remaining in the washed eggs. One may conclude, then, that retained amino acids do not influence the uptake of another amino acid, namely, valine. One or more of the amino acids in the added mixture evidently inhibit the uptake of valine when concurrently present in the medium. This was explored further with the individual amino acids and with fertilized as well as with unfertilized eggs.

3. *Effect of one amino acid on the uptake and incorporation of another*

(a) C^{14} -valine

Uptake, and incorporation into protein, of C^{14} -valine, C^{14} -glutamic acid and C^{14} -arginine by unfertilized and fertilized eggs were measured individually in the presence of an excess (*ca.* 3000 $\times$) of each of the other 19 "coded" amino acids. In some experiments the labeled amino acid was tested against the other 19 amino acids at the same time. In others about half of the amino acids were tested at one time, as noted in the legends for the figures. The results are represented graphically in Figures 1, 2 and 3. Tables III and IV present ratios of the average uptake of the labeled amino acid in the presence of the added C^{12} -amino acid to that in its absence. Ratios for incorporation are similarly presented. Table III includes results of an additional series of tests of incorporation (see footnote to table). In Figures 1, 2 and 3, for each experiment, the control values (indicated by NONE) are given at the top. These are followed by the values obtained for each of the added amino acids arranged in a decreasing (using the larger of each of the duplicate values) order of uptake.

For the unfertilized eggs the two experiments of Figure 1 with C^{14} -valine show marked (greater than 50%) inhibition of uptake by SER, ARG, ASN, GLN, ALA,

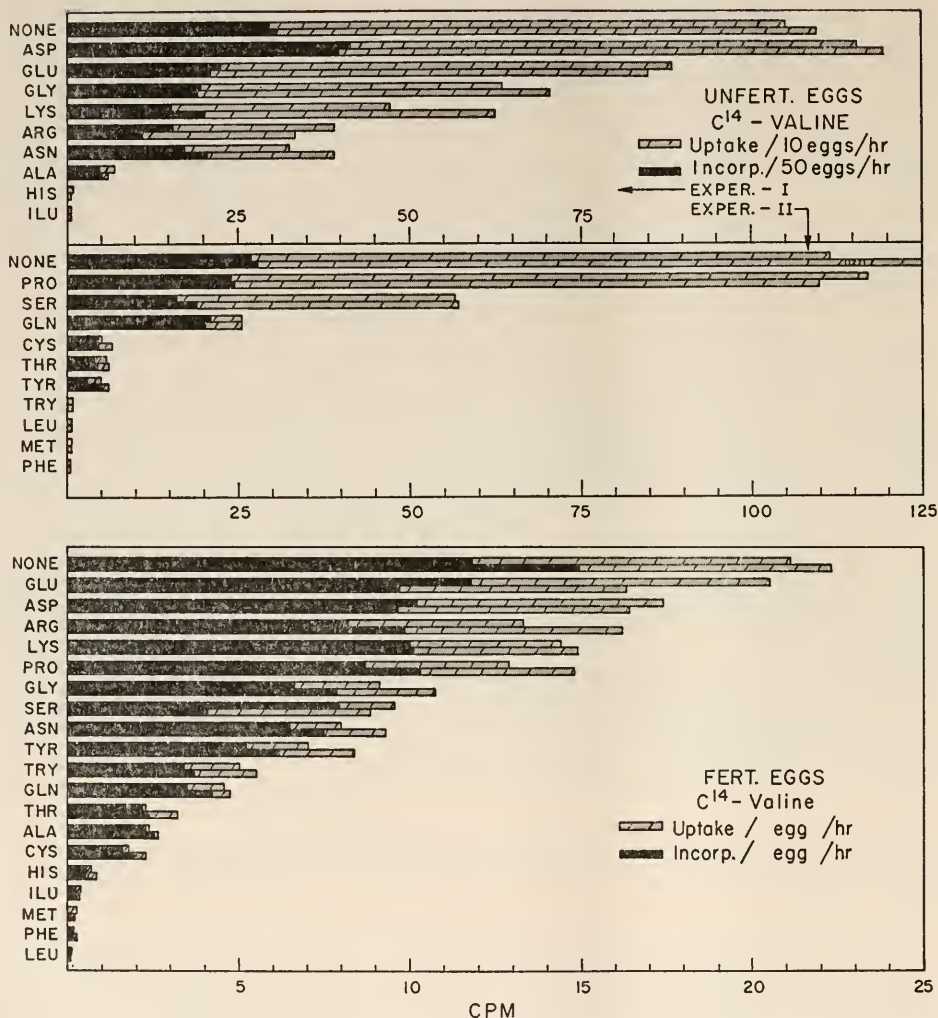


FIGURE 1. C^{14} -L-valine uptake, and incorporation into protein, by unfertilized and fertilized (one hour after fert.) eggs of *L. pictus* in presence of various individual C^{12} -L-amino acids. Incubation was for one hour in a total volume of 0.25 ml. of artificial sea water containing, per tube, 940 eggs (unfert., expt. I), 2860 eggs (unfert., expt. II) or 4314 eggs (fert.), and 0.83 μ c./ml. of the C^{14} -valine (sp. act. 208.5 c./M). The added amino acids were each at 0.012 M except TYR which was at 0.0004 M. The tests were all run in duplicate and the individual results are represented by each member of the pairs of bars. For the unfertilized eggs the tests were done with 9 of the C^{12} -amino acids (expt. I) on one day and with the remaining 10 (expt. II) on another occasion, using eggs from a different female. In the experiment with the fertilized eggs all 19 of the C^{12} -amino acids were tested at one time. For the entries to the left of the figure: None = no added C^{12} -amino acid; ALA = alanine; ARG = arginine; ASN = asparagine; ASP = aspartic acid; CYS = cysteine; GLU = glutamic acid; GLN = glutamine; GLY = glycine; HIS = histidine; ILU = isoleucine; LEU = leucine; LYS = lysine; MET = methionine; PHE = phenylalanine; PRO = proline; SER = serine; THR = threonine; TRY = tryptophan; TYR = tyrosine; VAL = valine.

CYS, THR, TYR, HIS, ILU, LEU, MET, PHE, TRY, listed in decreasing order of the average values, as given in Table III. For all except the first three of these the inhibition of uptake is greater than 75%, and for all except the first four the inhibition of uptake is greater than 90%. The values for degree of inhibition of incorporation are similar to those for inhibition of uptake for each of the tested amino acids except for GLN where incorporation is much less inhibited (24 to 30%) than is uptake (79%).

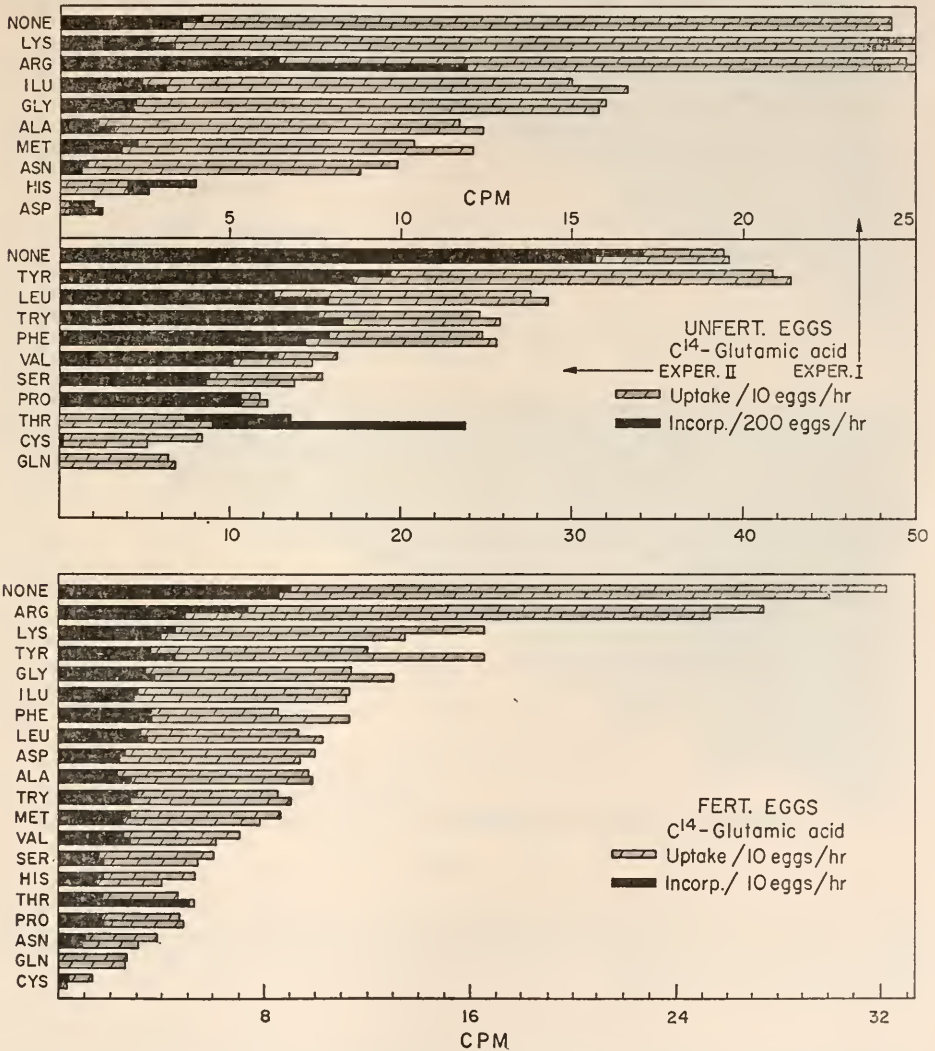


FIGURE 2. C^{14} -L-glutamic acid uptake and incorporation into protein; same description as for Figure 1, except that egg numbers were 3650 (unfert., expt. I), 1570 (unfert., expt. II) and 1190 (fert.).

For the fertilized eggs the amino acids that effect greater than 50% inhibition of both uptake and incorporation are the same as for the unfertilized eggs, except that the following are now brought just within this group: GLN (incorp.), GLY (uptake) and SER (incorp.). At the 75% level of inhibition the same amino acids

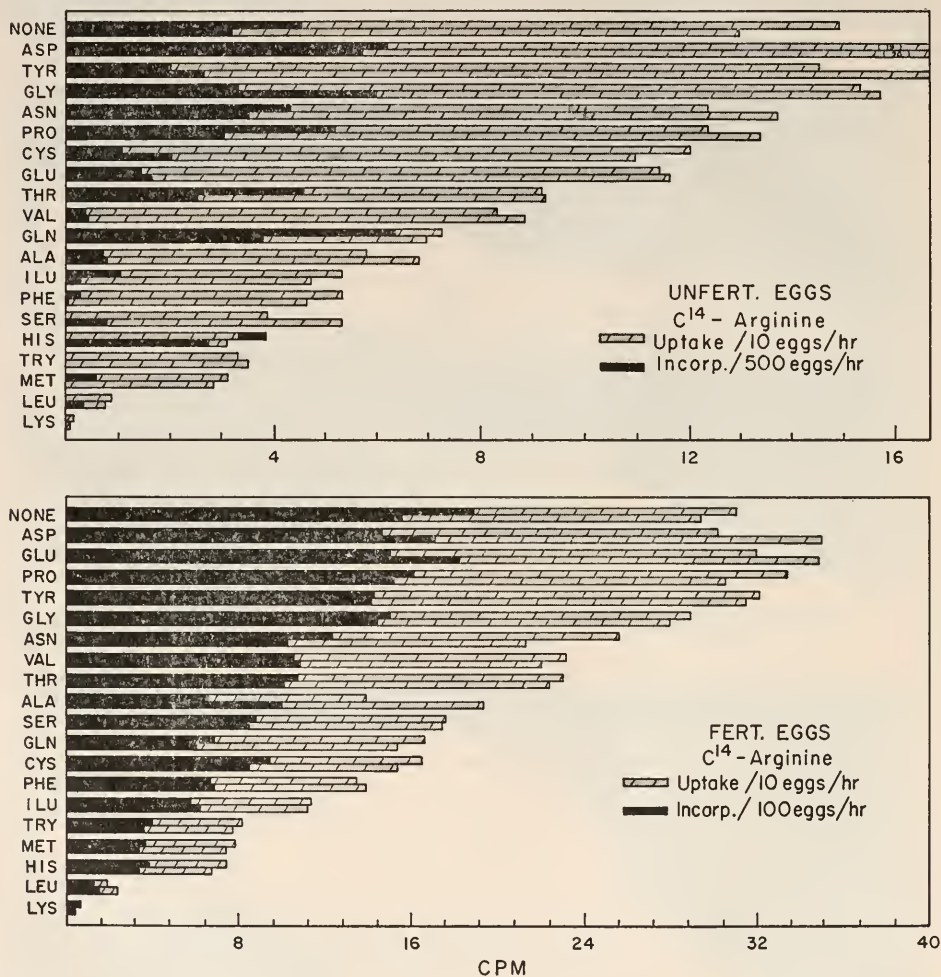


FIGURE 3. C^{14} -L-arginine uptake and incorporation into protein; same description as for Figure 1, except that egg numbers were 4170 (unfert.) and 4140 (fert.), that *L. anamesus* instead of *L. pictus* was used in the experiment with the unfertilized eggs and that the sp. act. of the C^{14} -arginine was 222 c./M.

are effective except for TYR (uptake and incorp.) and TRY (incorp.). Even at the 90% level of inhibition most of the inhibiting amino acids are the same as for the unfertilized eggs with respect both to uptake and incorporation, as comparisons of the values in Tables III and IV show.

The amino acids that effect the high (90% or better) degree of inhibition of uptake of C¹⁴-valine, both for unfertilized (ALA, CYS, THR, TYR, HIS, ILU, LEU, MET, PHE and TRY) and for fertilized (CYS, HIS, ILU, LEU, MET and PHE) eggs all belong to the neutral group, with the exception of HIS which is only weakly basic. This holds also for the inhibition of incorporation into protein.

TABLE III

Influence of individual amino acids on the uptake and incorporation into protein of a neutral, an acidic and a basic amino acid by unfertilized eggs of Lytechinus pictus, incubated for 1 hour at 20° C.*

"Competing" C ¹² -amino acid at 0.012 M	Ratios of cpm's for mixture of C ¹² - and C ¹⁴ -amino acid to cpm's for C ¹⁴ -amino acid alone								
	C ¹⁴ -L-Valine (3.9 × 10 ⁻⁶ M)			C ¹⁴ -L-Glutamic acid (3.9 × 10 ⁻⁶ M)			C ¹⁴ -L-Arginine (3.7 × 10 ⁻⁶ M)		
	Total uptake	Incorp. %	Incorp.**	Total uptake	Incorp.	Incorp.**	Total uptake	Incorp.	Incorp.**
Alanine	0.07	0.17	0.08	0.49	0.34	0.42	0.45	0.22	0.35
Arginine	0.34	0.44	0.54	1.07	2.36	1.31	—	—	—
Asparagine	0.33	0.64	0.83	0.04	0.20	0.09	0.93	1.07	0.70
Aspartic acid	1.09	1.35	0.92	0.01	0.29	0.12	1.38	1.65	1.94
Cysteine	0.05	0.15	0.01	0.18	0.07	0.69	0.81	0.47	0.83
Glutamic acid	0.80	0.71	0.81	—	—	—	0.83	0.43	0.95
Glutamine	0.21	0.76	0.70	0.17	0.00	0.20	0.51	1.42	0.90
Glycine	0.62	0.64	0.80	0.65	0.55	0.49	1.11	1.32	0.68
Histidine	0.00	0.01	0.46	0.09	0.85	0.26	0.23	0.92	0.39
Isoleucine	0.00	0.01	0.02	0.65	0.70	0.47	0.36	0.19	0.14
Leucine	0.00	0.01	0.01	0.72	0.44	0.55	0.06	0.07	0.09
Lysine	0.51	0.58	0.60	1.12	0.79	0.51	0.01	0.35	0.06
Methionine	0.00	0.02	0.01	0.46	0.52	0.26	0.22	0.07	0.01
Phenylalanine	0.00	0.01	0.01	0.65	0.46	1.15	0.35	0.15	0.34
Proline	0.95	0.89	0.89	0.31	0.33	0.52	0.93	1.15	0.87
Serine	0.48	0.63	0.71	0.37	0.27	1.13	0.33	0.08	0.23
Threonine	0.05	0.17	0.13	0.21	0.57	0.72	0.66	1.00	0.46
Tryptophan	0.00	0.03	0.04	0.64	0.49	0.52	0.24	0.00	0.20
Tyrosine***	0.05	0.15	0.12	1.08	0.56	0.66	1.12	0.65	0.68
Valine	—	—	—	0.40	0.35	0.53	0.62	0.10	0.39

* *Lytechinus anamesus* used in experiments with C¹⁴-L-arginine, columns 1 and 2. Eggs of this species resemble closely those of *L. pictus*.

** Separate experiment in which only incorporation into protein was measured.

*** At 0.0004 M.

(b) C¹⁴-Glutamic acid

For the unfertilized eggs all but five (ARG, GLY, ILU, LYS and TYR) of the 19 C¹²-amino acids cause greater than 50% inhibition of uptake or incorporation, or both, of the C¹⁴-glutamic acid. With the fertilized eggs all but one (ARG) do so. The 75% (or more) inhibition level with unfertilized eggs is attained by ASN, ASP (uptake), CYS, GLN, HIS (uptake), and THR (uptake). At this level, for the fertilized eggs, these same amino acids, except for ASP, are effective as are also HIS (incorp.), PRO, SER, THR (uptake) and VAL (uptake). At the 90%

level with the unfertilized eggs there are ASN (uptake), ASP (uptake), CYS (incorp.) and GLN (incorp.). With the fertilized eggs 90% inhibition is given only by CYS and GLN.

For the inhibition of uptake and incorporation of C^{14} -glutamic acid there again appears to be a relationship to type of amino acid. Thus strong inhibition is given by ASP, ASN and GLN which are all grouped in the acidic category. Only CYS and THR, of the neutrals, and HIS, of the basics, strongly inhibit uptake by the

TABLE IV

*Influence of individual amino acids on the uptake and incorporation into protein of a neutral, an acidic and a basic amino acid by fertilized eggs of *Lytechinus pictus*, 1 hour after fertilization.*

Incubation was for 1 hour at 20° C.

"Competing" C^{12} -amino acid at 0.012 M	Ratios of cpm's for mixture of C^{12} - and C^{14} -amino acid to cpm's for C^{14} -amino acid alone					
	C^{14} -L-Valine (3.9×10^{-6} M)		C^{14} -L-Glutamic acid (3.9×10^{-6} M)		C^{14} -L-Arginine (3.7×10^{-6} M)	
	Total uptake	Incorporation	Total uptake	Incorporation	Total uptake	Incorporation
Alanine	0.11	0.18	0.32	0.29	0.55	0.48
Arginine	0.68	0.67	0.85	0.68	—	—
Asparagine	0.40	0.52	0.11	0.11	0.78	0.65
Aspartic acid	0.78	0.74	0.31	0.27	1.08	0.92
Cysteine	0.09	0.14	0.03	0.04	0.53	0.52
Glutamic acid	0.85	0.81	—	—	1.11	0.97
Glutamine	0.21	0.29	0.08	0.01	0.53	0.38
Glycine	0.46	0.54	0.39	0.38	0.94	0.85
Histidine	0.04	0.05	0.15	0.17	0.24	0.21
Isoleucine	0.02	0.02	0.37	0.33	0.38	0.36
Leucine	0.01	0.01	0.32	0.37	0.08	0.08
Lysine	0.67	0.75	0.48	0.48	0.02	0.03
Methionine	0.01	0.02	0.27	0.30	0.25	0.23
Phenylalanine	0.01	0.01	0.32	0.38	0.45	0.40
Proline	0.64	0.71	0.16	0.21	1.06	0.91
Serine	0.42	0.43	0.18	0.20	0.58	0.50
Threonine	0.13	0.17	0.16	0.38	0.75	0.61
Tryptophan	0.24	0.27	0.29	0.33	0.26	0.21
Tyrosine*	0.36	0.43	0.46	0.46	1.05	0.83
Valine	—	—	0.21	0.30	0.75	0.62

* At 0.0004 M.

unfertilized eggs, and these same amino acids plus PRO, SER and VAL are similarly effective with the fertilized eggs.

(c) C^{14} -Arginine

Inhibition greater than 50%, for the uptake and incorporation, or both, of C^{14} -arginine, was obtained with all of the added C^{12} -amino acids with the exception of ASN, ASP, GLU, GLY, PRO, THR and TYR for the unfertilized eggs and, in addition, CYS and VAL for the fertilized eggs. At the 75% level of inhibition, only ALA, HIS, ILU, LEU, LYS, MET, PHE, SER, TRY and VAL for the

unfertilized eggs and HIS, LEU, LYS, MET and TRY for the fertilized remain inhibitory. At the 90% level of inhibition of uptake and/or incorporation LEU, LYS, MET, SER, TRY and VAL remain for the unfertilized eggs while only LEU and LYS are effective in the fertilized eggs.

It is evident that amino acids categorized as acidic did not significantly inhibit the uptake of C^{14} -arginine. In fact, ASP showed a slight enhancement of uptake and incorporation for the unfertilized eggs but this effect was not repeated with the fertilized eggs. Only a few neutral amino acids appreciably inhibited C^{14} -arginine uptake. On the other hand, both basic amino acids, LYS and HIS, showed strong inhibition for both unfertilized and fertilized eggs.

4. Effect of fertilization on uptake of amino acids and on incorporation into protein

Apart from the inhibitory effects of added amino acids, the data of Figures 1, 2 and 3 also permit incorporation to be compared with uptake with regard to the changes they undergo upon fertilization for C^{14} -valine, C^{14} -glutamic acid and C^{14} -arginine. This information is summarized in Table V. It is clear that uptake of

TABLE V

Effect of fertilization on uptake of amino acids and on incorporation into protein by eggs of Lytechinus (from data of Figures 1, 2 and 3; average values of cpm's per 10^3 eggs for 1 hour incubation)

	C^{14} -Valine			C^{14} -Glutamic acid			C^{14} -Arginine		
	Uptake (U)	Incorp. (I)	I/U	Uptake (U)	Incorp. (I)	I/U	Uptake (U)	Incorp. (I)	I/U
Unfertilized	11361	576	0.05	3172	91.7	0.03	1398	7.2	0.005
Fertilized	21738	13398	0.62	3116	901	0.29	3029	173	0.057
Fert./Unfert.	1.91	23	12.4	0.98	9.8	9.67	2.17	24	11.4

all three of these amino acids is high in the unfertilized egg. Upon fertilization there is an approximately two-fold increase in uptake of valine and of arginine, and no appreciable change in uptake of glutamic acid, at the stated external concentrations. The data for incorporation, however, show the usual great stimulation that occurs upon fertilization. In the present experiments these amount to 23- to 24-fold for valine and arginine, and 10-fold for glutamic acid. If incorporation is expressed in terms of uptake (columns 4, 7 and 10 of Table V) then the increase upon fertilization is of the order of 10-fold for all three amino acids, at the indicated concentrations and incubation time.

These comparisons are made apart from considerations of possible feedback inhibition of uptake, particularly in the fertilized eggs, and of possible effect of depletion of labeled amino acid from the medium. The data of Mitchison and Cummins (1966) show that with C^{14} -valine at a concentration of 0.14 mM there is no appreciable feedback inhibition of uptake by fertilized sea urchin eggs during a period of one hour. The concentration of valine in the present tests (0.0039 mM) is very much less than this. Therefore, feedback inhibition is unlikely. While similar information is not available for glutamic acid and for arginine the present

data would indicate that feedback inhibition is not likely to have occurred to any very appreciable extent in these experiments.

With regard to depletion of the labeled amino acid from the medium, calculations from the data presented in Figures 1, 2 and 3 show that the average concentrations in the medium at the end of the incubation period are reduced by approximately 2% for glutamic acid, 5% for arginine and 40% for valine. It is only for valine, then, that the value for uptake by the fertilized eggs may be appreciably affected by depletion of the label. The 40% reduction by the end of the incubation period would mean an approximately 20% average decrease in uptake, assuming linearity between uptake and concentration. This does not require altering the above statement of an approximately two-fold increase upon fertilization.

The external concentration employed in tests of valine-uptake is about one-fifth that found by Mitchison and Cummins (1966) to give maximum rate of uptake with fertilized eggs of *Paracentrotus lividus*. These workers, using concentrations well above that giving maximum rate of uptake for fertilized eggs, report a considerable increase in uptake upon fertilization. This may be estimated from their Figure 1 to amount to 15- to 30-fold. It would appear, then, that the amino acid concentrations at which the present measurements were made were in a range at which the uptake rate relative to the maximum attainable for the unfertilized egg was higher than that for the fertilized egg. This may also mean that the maximum rate is reached at lower concentrations for unfertilized than for fertilized eggs.

DISCUSSION

The present results provide information of use in studies of changes in protein synthesis upon fertilization and early development of sea urchin eggs. The demonstration by Mitchison and Cummins (1966), with fertilized sea urchin eggs, of the ability of one amino acid to inhibit the accumulation of another, has been confirmed, and the tests have been extended to include all twenty of the "coded" amino acids in the presence of a characteristic neutral, acidic and basic amino acid in both unfertilized and fertilized eggs. The analysis has shown that competition occurs primarily between amino acids that belong to the same group. However, these interrelationships are not exclusive and there is a certain degree of overlap.

As noted in the introduction there have been many studies (*e.g.*, Wilbrandt and Rosenberg, 1961; Scholefeld, 1961; Jacquez, 1961a, 1961b; Christensen, 1962, 1964; Christensen *et al.*, 1962; Oxender and Christensen, 1963; Johnstone and Scholefeld, 1965; Guroff *et al.*, 1964; Larsen *et al.*, 1964; Spencer and Brody, 1964; Adamson *et al.*, 1966; Alvarado, 1966) with cells of various other kinds of organisms, in which the influence of one amino acid on the uptake of another has been examined. Competition is found to occur largely within the separate groups but there are many exceptions. The same general conclusions apply to the results of our experiments.

The concentration of the competing amino acid in each of our tests with valine is many thousands of times higher than that at which, according to Mitchison and Cummins (1966), the maximum rate of uptake is attained. This is probably true also for glutamic acid and arginine although the plateau levels for these have not been determined. We may infer, then, that the experiments reveal all instances in which a particular amino acid has some appreciable ability to compete for entrance

into the cell with the three amino acids tested. The correlations between the uptake of an amino acid and the incorporation into protein are very good for the unfertilized eggs and even better for the fertilized eggs where the values are higher and variation is correspondingly lower. Thus, the inhibition that one amino acid effects on the incorporation of another evidently takes place at the uptake site. That this site operates independently of the sites of protein synthesis is suggested by the wide divergences between uptake and incorporation with respect to the changes in these properties that are observed upon fertilization.

As noted above, and as is summarized in Table V, the unfertilized eggs exhibit a relatively high capacity for uptake of the three test amino acids, and the increase upon fertilization is evidently rather small. The high amino acid uptake rate of the unfertilized egg contrasts with other uptake systems studied in sea urchin eggs. For example, phosphate uptake (Whiteley, 1949; Whiteley and Chambers, 1961) and nucleoside uptake (Nemer, 1962; Piatigorsky and Whiteley, 1965; Mitchison and Cummins, 1966) are very strongly suppressed, as is the transport of many other substances in the unfertilized sea urchin egg (*cf.* Monroy, 1965; Rothschild, 1956).

Apart from the theoretical considerations that are of interest in the transport of amino acids into cells, one may utilize the data presented here, in combination with measurements of the maximum rates at which the various labeled amino acids are incorporated into protein, to specify the more effective mixtures of amino acids for labeling nascent protein in sea urchin eggs. Measurements of rates of incorporation of individual labeled amino acids as a function of concentration have been made on eggs of *Lytechinus* at one hour after fertilization (Tyler, 1965b and unpublished). From these measurements the presently available values for the (approximately) maximum incorporation into protein, in $m\mu$ moles incorporated in one hour by 10^4 eggs (and the values for the external concentrations, in μ moles/ml., at which maximum or near maximum incorporation is first attained given in parentheses) are as follows: ALA-2.3(30), ARG-2(>60), ASN-0.5(2.8), ASP-1.7(>1.9), CYS-CYS-0.3(sat. in s.w.), GLU-4.5(40), GLN-2.6(0.1), GLY-1(3.8), HIS-0.5(0.1), ILU-3.8(0.3), LEU-1.6(0.03), LYS-0.5(120), MET-0.2(0.03), PHE-0.4(0.1), PRO-1(>0.24), SER-2(8), THR-2.5(1.0), TRY-2(sat. in s.w.), TYR-0.2(0.4), and VAL-3.3(0.1).

Depending upon how the various parameters are evaluated and combined a number of highly effective mixtures may be formulated. The general procedure is to maximize incorporation into protein while minimizing effects of competition among the amino acids. It is assumed that the labeled amino acids would be available at about the same specific activity. One example of a group of amino acids that would yield high radioactivity of nascent protein is: ILU, ARG, GLU and PRO. The addition of other amino acids would tend to reduce incorporation by virtue of competition of uptake. However, one may substitute for each of these certain other "competing" amino acids that have reasonably high values of incorporation when tested individually. For example, if VAL were substituted for ILU there would not be a very great over-all change in the values for incorporation given by the mixture. Similarly ASP could be substituted for GLU without large effect, as could LYS for ARG. Obviously, there are many more complex mixtures and substitutions that might be formulated, but since the present tests were made

with only 57 of the 380 possible combinations, further assessment of the most effective mixtures does not seem warranted at this time.

SUMMARY

1. Tests were made of the uptake and incorporation into protein of a neutral (C^{14} -valine), an acidic (C^{14} -glutamic acid) and a basic (C^{14} -arginine) amino acid in the presence of a mixture of other amino acids and in the presence of a great excess (3000-fold) of each of the other "coded" amino acids by unfertilized and fertilized eggs of *Lytechinus pictus*.

2. The results showed competition occurring principally among amino acids belonging to the same group. For C^{14} -valine the amino acids that effected strong inhibition (90% or greater) of uptake with unfertilized eggs were ALA, CYS, THR, TYR, HIS, ILU, LEU, MET, PHE and TRY, and with fertilized eggs were CYS, HIS, ILU, LEU, MET and PHE. For C^{14} -glutamic acid 90% inhibition of uptake was given by ASN and ASP with unfertilized eggs and by CYS and GLN with fertilized eggs. Finally, strong inhibition of C^{14} -arginine uptake was demonstrated by LYS and LEU with both unfertilized and fertilized eggs. Similar results were obtained in the corresponding tests of incorporation into protein. The inhibitory effects on incorporation are, then, attributable to competition for uptake.

3. In contrast to the relatively low capability of the unfertilized egg to incorporate amino acid into protein it possesses a relatively high ability to accumulate amino acids from the surroundings. For C^{14} -valine and C^{14} -arginine, the uptake rate by the unfertilized egg was approximately half of that of the fertilized egg, while for C^{14} -glutamic acid the pre- and post-fertilization rates of uptake were approximately the same.

4. The percentage of accumulated C^{14} -amino acid that was incorporated in one hour into protein in these experiments with valine, glutamic acid and arginine was 5, 3 and 0.5, respectively, in the unfertilized eggs and 60, 30 and 6, respectively, in the fertilized eggs. When expressed in terms of uptake, and assuming no large change in the pool of free amino acid in the egg, there is an approximately 10-fold increase in incorporation into protein upon fertilization for each of these three amino acids.

5. The results, also, enable formulations to be made of the kinds of combinations of labeled amino acids that would be the more highly effective in labeling nascent proteins of sea urchin eggs. One such combination would be ILU, ARG, GLU and PRO with each of these being replaceable by certain alternative "competing" amino acids as indicated in the text.

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ERRATA

In the paper by Hayward and Ball in volume 131, number 1, of THE BIOLOGICAL BULLETIN, the first lines on page 100 should read as follows: "of any added stimulation, the mean value for the rate of oxygen consumption of brown adipose tissue was 2.33 and 3.29 times that of liver and heart, respectively. The addition of epinephrine caused an average increase of 350% in the respiratory rate of brown adipose tissue, and was without effect upon liver or heart slices. In . . ."

On page 102, line 7 of the Summary, the value should read "1374 μ l. O₂/100 mg. fresh tissue/hr."

THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

DISTRIBUTION OF URCEOLARIA SPINICOLA (CILIATA, PERITRICHIDA) ON THE SPINES OF THE SEA URCHIN STRONGYLOCENTROTUS DROEBACHIENSIS

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Most of the described species of *Urceolaria* occur epizoically on various fresh-water and marine invertebrates (Hirshfield, 1949). Probably *U. patellae*, from the ctenidia of the European limpet *Patella vulgata*, and *U. mitra*, from the external surface of fresh-water triclads, are the best known, owing to the ecological studies of Brouardel (1941, 1947) and Reynoldson (1950, 1955). The only species known at present from echinoids is *U. spinicola* Beers, 1964, which occurs in abundance on the spines and pedicellariae of *Strongylocentrotus droebachiensis*, at least in the waters adjoining Mount Desert Island, Maine. Since the ciliate appears to be obligately epizoic on the urchin, its geographic range is probably coextensive with that of the host. In general, *U. spinicola* has the form of a short cylinder, which measures about 60 μ in diameter and 25 μ in height. By means of its specialized basal disc, it adheres firmly to the spines and pedicellariae, although it is capable of limited locomotion, either by sliding along the substratum or, less commonly, by swimming freely in the medium.

The preceding study (Beers, 1964) was concerned chiefly with the structure and identification of the ciliate and with its actual occurrence on the urchins of Mount Desert Island. Its distribution on the spines received only incidental mention, although it presented some remarkable features. The evidence indicated, for example, that short spines had many more urceolarias per spine than long ones, and that the ciliates attached to long spines were concentrated on the proximal halves of the spines. The reference to long and short spines does not mean primary and secondary ones. The spines of any specimen of *S. droebachiensis* differ greatly in length, but the intergrades between the extremes are practically countless. Thus, the spines cannot be separated into two categories (Hyman, 1955, p. 424). In view of the fundamental similarity of the spines, any differential distribution of *U. spinicola* on their surfaces assumes added interest. Therefore, a more thorough study of the distribution was undertaken in the summer of 1965, again on the

urchins of Mount Desert Island. The results are presented in the present paper, which deals mainly with the following aspects of the urchin-ciliate association: (1) the occurrence of the ciliate on spines from different regions of the urchin test; (2) the density of the ciliate population (intensity of epifauna) on urchins of different sizes; (3) the occurrence of the ciliate on spines of different lengths; and (4) its distribution on individual spines. Once the distribution on the spines is definitely established, an analysis of the factors responsible for such distribution can be attempted, but this aspect of the study is deferred for the present. Any consideration of the distribution of the urceolarias on the pedicellariae is likewise deferred.

MATERIAL AND METHODS

From June 15 to August 25, 1965, specimens of *S. droebachiensis* were collected as needed from the inshore waters of Mount Desert Island. They were taken from three localities: Laboratory Point, Bartlett Narrows and Long Ledge. Laboratory Point means the waters of Frenchman Bay adjacent to the Laboratory area. In the summer the Bay is relatively calm and littoral urchins are subjected to the minimum of wave action. Thus, their spines show very little weathering at the tips. Although the mean tidal range of the Bay amounts to 3.25 m., the amount of organic matter in the water and the bacterial count were evidently high in 1965, since much of the Bay was closed to the taking of mussels and clams for table use. In general, the waters of Bartlett Narrows, a strait in Blue Hill Bay, are likewise free of turbulence and in 1965 they were relatively uncontaminated. Long Ledge, well removed from Laboratory Point and Bartlett Narrows, presents a somewhat different habitat. The waters are quite uncontaminated, the Ledge is exposed to the winds, and a surf is constantly present. Thus, the long spines of inshore urchins are much eroded distally.

Counts of the urceolarias were made on detached fresh spines. A small piece of the test was excised from a recently collected urchin and removed to a watch glass of sea water under the dissecting binocular, with the spines uppermost. The piece was held down by a blunt needle, the tip of a small scalpel was brought against the base of a spine, and the spine was detached by a quick movement of the scalpel. When a sample of several contiguous spines was desired, the spines were detached in turn, beginning at the margin of the piece. The number of urceolarias dislodged by the procedure was negligible.

It is practically impossible to count the urceolarias *in situ* on a spine, largely because of its opacity. In order to count them, the detached spines were transferred in groups of five or ten to a watch glass of distilled water. When a fresh spine is immersed in distilled water, any urceolarias on it are immediately immobilized and after 3-5 min. they become detached. If the spine is shaken gently with forceps, they drop to the bottom of the watch glass, where they can be counted accurately.

With reference to the distribution of *U. spinicola* on the urchin, the following three regions of the test were distinguished: a circumoral region, meaning the somewhat flattened surface which is normally in contact with the substratum; an ambital or circumferential region; and an aboral region, meaning the expanse between the ambitus and the periproct.

The ages of certain of the urchins were estimated from the diameter of the test, following the data summarized by Swan (1961, Table IV). In the presentation of the results, comparisons will be made occasionally between the numbers of urceolarias in two groups. If the larger number exceeds the smaller by one-third or more, the difference is judged to be significant. Minor comments on methods will be supplied as needed.

RESULTS

1. *Occurrence of U. spinicola on spines from different regions of the urchin test; intensity of epifauna on urchins of different sizes*

Urchins of various sizes (measured by the diameter of the test) were examined from each of the three localities. Their respective sizes are listed in column 1 of Table I and their corresponding ages in column 2, in so far as estimates of age are available. Most of the sizes represent recognized year-classes, but some urchins of undetermined age are also included. Five urchins of each of nine sizes were examined from Laboratory Point. Unfortunately, urchins 9–18 mm. in diameter were unavailable at Bartlett Narrows and Long Ledge, but five of each of the

TABLE I

Occurrence of U. spinicola on urchins (S. droebachiensis) of different sizes from three localities on Mount Desert Island, Maine (Laboratory Point, Bartlett Narrows and Long Ledge). Summer 1965

Diameter of test in mm.	Age in years from time of settling	Locality	Average number of urceolarias per spine			
			Circumoral region	Ambital region	Aboral region	Entire urchin
9–10	1	Lab. Point	1.3	1.0	0.5	0.9
12–14	?	Lab. Point	2.3	1.7	0.7	1.6
16–18	?	Lab. Point	18.4	19.1	10.1	15.9
24–26	2	Lab. Point	20.9	23.7	12.6	19.1
30–38	?	Lab. Point	28.2	32.4	13.9	24.8
40–42	3	Lab. Point	37.8	35.1	26.4	33.1
46–54	4	Lab. Point	43.1	41.6	24.3	36.3
55–60	5	Lab. Point	22.9	26.8	16.2	22.0
62–74	6+	Lab. Point	2.7	3.4	2.8	3.0
24–26	2	Bart. Nar.	9.8	8.2	4.5	7.5
30–38	?	Bart. Nar.	22.1	25.8	9.6	19.2
40–42	3	Bart. Nar.	11.0	9.2	3.6	7.9
46–54	4	Bart. Nar.	10.5	8.2	4.1	7.6
55–60	5	Bart. Nar.	1.8	1.2	0.6	1.2
62–70	6+	Bart. Nar.	0.8	0.6	0.6	0.7
24–26	2	Long Ledge	4.7	5.2	4.6	4.8
30–38	?	Long Ledge	4.0	2.9	2.2	3.0
40–42	3	Long Ledge	2.1	1.9	0.7	1.6
46–54	4	Long Ledge	2.3	2.3	1.4	2.0
55–60	5	Long Ledge	2.6	2.4	1.0	2.0
62–72	6+	Long Ledge	0.8	0.5	0.4	0.6

remaining sizes were examined from these areas. The average number of urceolarias per spine was determined from a spine-sample taken from each of the three regions of each urchin. Such a sample consisted of any ten contiguous spines from an excised piece of test. Thus, each entry in columns 4, 5 and 6 of Table I represents an average based on 50 spines. It is understood that the spines of any sample varied considerably in length (usual range, 1.0–15.0 mm., but reduced to 0.5–5.0 mm. in samples from small urchins).

Turning to Table I, consider the average number of urceolarias per spine on different regions of the test, beginning with the urchins from Laboratory Point. In any of the nine size-classes, the average number of ciliates per spine was approximately the same on the circumoral and ambital regions (columns 4 and 5). For example, in size-class 24–26 mm. the average numbers were 20.9 and 23.7, respectively (no significant difference). On the other hand, the average number on the aboral spines (column 6) was decidedly smaller in all the size-classes, with one exception—the class consisting of the largest urchins (62–74 mm.), which had very few ciliates per spine, regardless of the region. In general, the foregoing comments also apply to the urchins from Bartlett Narrows, although the average number of ciliates per spine was smaller without exception. With reference to the Long Ledge urchins, the ciliate populations were extremely sparse and the average number of urceolarias per spine was therefore much reduced. Indeed, the ciliate counts were so small that comparisons between the respective regions of the test are scarcely practicable. Nevertheless, the general features of the distribution were in agreement with those already described.

Referring again to Table I, consider the average number of urceolarias per spine on urchins of different sizes; that is, the intensity of epifauna of the entire urchin (column 7, each entry of which is based on a total of 150 spines). With reference to the urchins from Laboratory Point, the average number of ciliates per spine increased with the size of the urchin, until a diameter of 46–54 mm. (or an age of about 4 years) was attained. On urchins larger than these, the number decreased abruptly. The scarcity of urceolarias on urchins 62 mm. or larger in diameter (presumed to be at least 6 years of age) was remarkable. Indeed, on many urchins of this size it was impossible to find any urceolarias, either on the spines or pedicellariae. In general, the foregoing remarks also apply to the urchins from Bartlett Narrows, although the average number of ciliates per spine was consistently smaller and the maximal number occurred on urchins 30–38 mm. in diameter, some of which were probably 3 years of age. On the Long Ledge urchins the average number of urceolarias per spine was small, and comparisons between successive sizes are therefore less meaningful. Nevertheless, the trend in the intensity of epifauna agreed with that already mentioned.

Spine-samples from the ambulacral and interambulacral areas of certain urchins were also examined comparatively, although the results are not presented in tabular form. Without exception, the average number of urceolarias per spine was essentially the same on the two areas. For example, on a 41-mm. urchin from Laboratory Point, the average number per spine was 27.3 on an ambulacral area and 26.7 on an adjacent interambulacral area, based on a sample of 50 spines removed at random from each area. Evidently the presence of the tube feet does not affect the occurrence of the ciliate.

In summary, the results show (1) that *U. spinicola* is more abundant on the circumoral and ambital spines than on the aboral ones; (2) that it occurs in equivalent numbers on the ambulacral and interambulacral areas; (3) that the density of the ciliate population increases gradually as the urchin grows and attains its maximum on urchins 40–54 mm. in diameter; and (4) that the density decreases markedly on urchins 62–74 mm. in diameter, many of which bear no urceolarias whatsoever.

2. *Occurrence of U. spinicola on spines of different lengths from three regions of the urchin test*

Considerable numbers of spines were detached from each of the three regions of five urchins (diameter, 40–42 mm.) from Laboratory Point, and the number of urceolarias per spine was recorded. These records supplied numerous counts for

TABLE II

Occurrence of U. spinicola on spines of different lengths from three regions of the urchin test. Number of urchins, 5. Diameter of test, 40–42 mm. Spine-sample, 10; namely, 2 spines of each length from each region of each urchin

Spine length in mm.	Average number of urceolarias per spine			
	Circumoral region	Ambital region	Aboral region	Entire urchin
0.6–0.9	8.8	7.4	10.2	8.8
1.0–1.9	29.5	31.3	21.0	27.3
2.0–2.9	28.9	32.4	18.4	26.6
3.0–3.9	42.9	30.3	14.9	29.4
4.0–4.9	28.8	21.5	10.1	21.5
5.0–5.9	14.6	13.4	7.1	11.7
6.0–6.9	8.0	6.4	3.8	6.1
7.0–7.9	6.3	4.6	4.5	5.1
8.0–8.9	5.8	3.9	1.6	3.8
9.0–9.9	3.1	2.5	0.6	2.1
10.0–10.9	*	2.7	1.8	2.3
11.0–16.0	*	2.8	1.2	2.0

* None of this length present.

spines of many different lengths. Representative data on the relation of the number of urceolarias to the length of the spine are presented in Table II. For descriptive purposes, most of the spines will be treated as “short” and “long” ones. Although these terms are relative, they are nonetheless useful. Spines 1.0–4.9 mm. in length, which comprise about 80% of the spines on urchins 24 mm. or more in diameter, will be called short spines, whereas spines 5.0 mm. or more in length, which comprise about 15%, will be called long spines. Spines shorter than 1.0 mm., which make up the remainder, are therefore uncommon on such urchins.

Reference to Table II shows that the smallest spines (length, 0.6–0.9 mm.) of all three regions had relatively few ciliates per spine. The average number varied from 7.4 to 10.2, and the average for the entire urchin (based on 30 spines) was 8.8. Presumably most of these spines were immature ones which had not acquired their full complement of urceolarias. Spines 1.0–4.9 mm. in length, on the other

hand, had the largest numbers of ciliates per spine; for example, the average number on the circumoral spines varied from 28.8 to 42.9. Then, spines 5.0 mm. or more in length had decreasing numbers of ciliates, and in general the average number per spine varied inversely with the length of the spine. In agreement with the data of Table I, spines from the circumoral and ambital regions had approximately equal (and maximal) numbers of ciliates, whereas those from the aboral region had fewer, although certain exceptions appear in Table II.

In general, the distribution of urceolarias shown in Table II was typical of urchins 24–60 mm. in diameter from Laboratory Point. For example, 50 short spines detached at random from five 25-mm. urchins had an average of 17.2 urceolarias per spine whereas 50 long ones had only 4.4 per spine. Similarly, 50 short spines from five urchins 55–58 mm. in diameter had 29.4 ciliates per spine, and 50 long ones had only 4.8. Thus, the results show conclusively that the short spines of urchins 24–60 mm. in diameter bear many more urceolarias per spine than the long ones.

Some further aspects of the urchin-ciliate association can be mentioned at this point. With respect to any individual urchin, the number of urceolarias on the spines of a particular length is extremely variable. For example, on ten ambital spines of length 2.0–2.9 mm. from a 50-mm. urchin, the number varied from 12 to 57; on ten spines of length 6.0–6.9 mm., from 1 to 15; and on ten of length 10.0–16.0 mm., from 0 to 9. It is evident, furthermore, that the number will vary with the intensity of epifaunation of the host. The largest number of urceolarias found on any spine in the entire study was 157 on an ambital spine 3.2 mm. long from a 31-mm. urchin. If an urchin bears a somewhat dense urceolaria population (of the degree indicated in Table II), ciliates will be found on practically every short spine, including those of the periproct, but their occurrence on long spines is unpredictable. It is a remarkable fact, which is at present unexplained, that urceolarias are absent on many of the longest spines (length, 10.0–16.0 mm.), even though the urchin as a whole harbors a dense population.

3. *Distribution of U. spinicola on individual spines of different lengths*

It has been shown that short spines bear significantly more urceolarias per spine than long ones, but there is a further peculiarity in the distribution. Briefly, the ciliates are not always distributed uniformly along the spine; on long spines they are concentrated on the basal (proximal) half. The regional distribution on individual spines was studied by cutting detached spines in half transversely and counting the urceolarias on the respective halves. A 45-mm. urchin from Laboratory Point was selected for special examination, since such urchins usually had undamaged spines and substantial epifaunations.

The counts compiled from various spine-samples from this urchin are summarized in Table III. From this Table it is seen that spines 0.6–0.9 mm. in length from any of the three regions had approximately equal numbers of urceolarias on the basal and distal halves. Likewise, spines 1.0–1.9 and 2.0–2.9 mm. in length had equivalent numbers on their respective halves. Spines 3.0–3.9 mm. in length, on the contrary, had approximately three times as many on the basal half as on the distal, and spines 4.0–4.9 mm. in length showed a still greater difference in numbers between the halves. Finally, spines 5.0 mm. or more in length had on the basal half

many times the number on the distal half. The spines of two additional urchins, a 34-mm. specimen from Bartlett Narrows and a 31-mm. one from a lobster trap in 11 m. of water in Frenchman Bay, were subjected to a similar analysis with results in full agreement with those of Table III.

With reference to the long spines, the data as presented in Table III are inadequate to show the true distribution on them. For example, on spines 5.0–6.9 mm. in length, most of the urceolarias of the basal half were actually restricted to the basal third, and on spines 7.0 mm. or greater in length, to the basal fourth or even the fifth. Unfortunately, lack of time prevented me from cutting such spines into four parts and counting the ciliates on the respective quarters. Thus, the

TABLE III
Distribution of U. spinicola on individual spines (basal and distal halves, respectively) from a 45-mm. urchin. Spine-sample: 5 of each length from each region

Spine length in mm.	Average number of urceolarias on circumoral spines		Average number of urceolarias on ambital spines		Average number of urceolarias on aboral spines	
	Basal half	Distal half	Basal half	Distal half	Basal half	Distal half
0.6–0.9	8.2	6.8	8.8	10.2	9.0	7.6
1.0–1.9	11.4	12.2	16.8	18.8	12.6	11.4
2.0–2.9	21.2	20.4	22.4	18.0	12.4	11.2
3.0–3.9	34.0	11.2	35.8	9.6	7.6	2.6
4.0–4.9	27.2	7.8	21.4	6.2	4.6	0.0
5.0–5.9	10.8	0.2	9.0	0.0	5.8	0.4
6.0–6.9	5.0	0.2	6.6	0.2	3.0	0.2
7.0–7.9	4.4	0.4	2.2	0.0	1.4	0.0
10.0–15.0	*	*	0.8	0.0	0.4	0.0

* None of this length present.

statement is based merely on an inspection of the spines and not on actual counts, but I believe that it is nonetheless correct.

In summary, the data of Table III permit the following generalization: on spines 3.0 mm. or less in length, the urceolarias are distributed uniformly along the length of the spine; on spines longer than 3.0 mm., they are largely restricted to the basal half of the spine.

4. Consideration of some factors affecting the distribution of *U. spinicola* on individual spines

With reference to such factors, various possibilities suggest themselves. For example, does the distribution coincide with the ciliation of the spines? Hyman (1955, p. 438) points out that in echinoids the epidermis of the spines is "more or less ciliated" and that "the ciliation tends to disappear with age except around the spine base. . . ." In my experience with the spines of *S. droebachiensis*, however, carmine particles are swept energetically toward the distal ends of all the spines regardless of their length, indicating that much, if not all, of the spine surface is ciliated. Thus, the evidence indicates that the absence of urceolarias on the distal portions of long spines does not result from the absence of cilia. Furthermore,

specimens of *U. spinicola* which have been gently brushed off the spines are capable of adhering firmly to various non-ciliated surfaces, such as glass, metal and granite. Although these observations are not extensive, they show at least that a ciliated surface is not necessary for the firm attachment of *U. spinicola*.

A second possibility affecting distribution relates to the constant movements of the pedicellariae and spines; that is, does contact of the pedicellariae with the long spines or contact of such spines with one another limit the distribution of urceolarias to the basal portions? The movements of the pedicellariae, spines and attached ciliates can be readily observed on an excised piece of test. The stalks of the pedicellariae, especially those of the triphyllous and tridentate ones, vary considerably in length, and in their movements the outer surfaces of the jaws commonly rub against spines of various lengths. Indeed, the jaws of the shorter pedicellariae frequently come in contact with the ciliates on spines 2.0–3.0 mm. long. When touched, the urceolarias move away from the area of contact, but they quickly resume their former distribution. In view of their abundance on such spines, it is evident that their distribution is not adversely affected by contact with the pedicellariae. Spines may likewise touch the ciliates on other spines, but with little more than a temporary disturbance of the distribution. It is unusual for the jaws of a pedicellaria actually to seize a spine and thereby injure the ciliates.

If it is assumed, nevertheless, that mechanical contact affects the distribution unfavorably, one might expect the ciliates on the bases of long spines to distribute themselves uniformly when the spines are detached and thereby isolated from one another. To ascertain whether the distribution changes under such conditions, 12 spines 3.6–4.8 mm. in length, which had urceolarias on their basal halves only, were detached from a 35-mm. urchin and transferred to two Syracuse watch glasses of filtered sea water (six spines in 8 ml. in each watch glass; water changed daily; normal temperature of 14° C. maintained). The average number of ciliates per spine (counted at the end of the experiment) was 32. The general distribution of the ciliates on the respective quarters of each spine was recorded daily. In such an experiment it is difficult to compile quantitative data, since it is impossible to count accurately the number of urceolarias on any part of a relatively opaque spine. Fortunately, such data were not needed, for the changes in the original distribution were almost negligible. For example, after 3 days conditions in the watch glasses were as follows: cilia still active on the spines (epidermis living); urceolarias firmly attached (none swimming freely), moving slightly on the spine surface (normal behavior); two urceolarias on the penultimate quarter of each of two spines; none on the distal quarters; the remainder on the basal halves as originally. The experiment was discontinued 2 days later, when conditions were as follows: 29 ciliates detached and motionless near their respective spines; one swimming freely; 14 on the penultimate and distal quarters of certain spines; the remainder, totaling 340, still attached to the basal halves.

The experiment was repeated, using 12 spines 4.6–6.4 mm. in length from a 42-mm. urchin which carried an especially heavy epifauna. The average number of urceolarias per spine was 31. On eight of the spines, the ciliates were restricted to the basal quarter; on the remaining four, to the basal half. After 4 days the original distribution was unchanged, except for four ciliates on the penultimate quarter of one spine. Two days later, when the ciliates were beginning

to die and detach, this distribution still prevailed. It is evident, therefore, that when spines are detached and isolated from contact with other spines or pedicellariae, the distribution of the urceolarias undergoes no significant change.

DISCUSSION

Since the presence of *U. spinicola* on its host was demonstrated somewhat recently, there has been little opportunity for an intensive study of the host-ciliate relationship. Nevertheless, certain features can be discussed briefly.

Transmission from host to host. In *U. patellae*, Brouardel (1947) observed that a very small percentage of the urceolarias left the limpet spontaneously from time to time and swam freely. Somewhat larger numbers detached when the host was in an unhealthy or moribund condition, and agitation of the medium facilitated detachment. Some of the free-swimming urceolarias survived for 6–8 hr. in sea water, and urceolaria-free limpets acquired ciliates when immersed in the water. Reynoldson (1950) concluded that *U. mitra* was dispersed when small populations occasionally assumed a free-swimming habit.

My efforts to induce *U. spinicola* to leave its host and disperse in the medium were notably unsuccessful. Its persistent adhesion to detached spines has been mentioned. Its behavior was also studied from day to day on excised pieces of test and on eviscerated whole tests. A few of the ciliates detached and swam briefly, but the number was insignificant, and the remainder perished *in situ*. Agitation of the medium, whether by vigorous stirring or by directing a stream of sea water on the urchin, was also ineffective. When a strong stream of water from a small glass nozzle was directed on a spine, the urceolarias merely retreated to the opposite side of the spine.

Probably the natural method of dispersal can be determined only by studying the association throughout the entire year. In *U. patellae*, Brouardel (1941) observed well-defined seasonal variations in the density of population, which was minimal in April and maximal in September and October. He found that dividing individuals were relatively numerous in May, but very scarce in January. In *U. spinicola*, the population appears to be relatively stable in the summer months. Dividing individuals are scarce—a fact reported earlier (Beers, 1964) and confirmed in the present study—and the population density, judged by counts per spine, seems to be as high in mid-June as in late August. Evidently *U. spinicola* in summer is physiologically specialized for continued adhesion to the host and not for dispersal. Presumably dispersal to new hosts occurs at other times of the year.

Population density in relation to habitat of the host. In *U. mitra*, Reynoldson (1955) found that fluctuations in the ciliate population were directly correlated with changes in the bacterial population of the water. Since *U. spinicola* feeds primarily on bacteria, its high incidence on the urchins of Frenchman Bay is attributed to an abundance of bacterial food. Similarly, its low incidence on the littoral urchins of Long Ledge is attributed largely to a scarcity of food, although the abrasive action of the surf, which erodes the spines, probably reduces the ciliate population through mechanical injury. Presumably the waters of Bartlett Narrows are intermediate with respect to the availability of food.

For the present I am unable to explain why *U. spinicola* is less abundant on the aboral surface of the host than elsewhere. My earlier statement (1964) to the

effect that "it is found very sparingly on the spines and pedicellariae of the equator" is incorrect; evidently it resulted from the examination of inadequate samples.

Distribution on individual spines. Probably the most remarkable feature of the distribution of *U. spinicola* concerns its abundance on short spines, its scarcity or absence on long spines, and its concentration on the basal portions of such spines, when it is present. Attempts to correlate the distribution with the ciliation of the spines or with certain mechanical factors, such as contact with other spines, were unsuccessful, as has been said. It may be argued that the distribution results from an avoidance of strong water currents which sweep across the surface of the urchin in its natural habitat. Actually, such currents are absent at Laboratory Point and elsewhere in Frenchman Bay, except in restricted channels of strong tidal flow. Furthermore, urchins may be kept in good health for many days in an aquarium containing gently running sea water, provided they are supplied with suitable food, such as *Laminaria*. In the absence of strong water currents, these urchins retain their urceolarias in abundant numbers for at least 10 days, and the distribution on the spines undergoes no observable change. Finally, large urchins (diameter, 62–74 mm.) occupy the same natural habitat as smaller ones. Yet *U. spinicola* is very scarce or even absent on the spines and pedicellariae of large urchins. It is evident, therefore, that its distribution cannot be related to water currents.

The availability of bacterial food on the surface of the urchin remains to be considered. It may be argued that suitable food is more plentiful near the surface of the urchin than at the free extremities of the long spines. If the correctness of this proposition is conceded, it still does not explain the distribution on the spines. For example, a long spine is usually surrounded by a group of short spines. Yet *U. spinicola* is abundant on the short spines, but scarce or absent on the base of the adjacent long spine. In this connection the scarcity or absence of the ciliate on large urchins must be mentioned again. Presumably bacterial food is quite as abundant on the surface of these urchins as on smaller ones.

It is evident that an explanation of the distribution must be sought in factors other than those already mentioned. For the present I am disposed to conclude that the distribution is related to certain intrinsic properties of the spines themselves, perhaps to the histological structure of the spine epidermis. The conclusion implies that the spine surface is not a uniform substratum. Although ciliated columnar cells predominate in the epidermis of echinoids, various types of gland cells are also present, as Hyman (1955, p. 438) indicates. It is possible that the distribution of *U. spinicola* is correlated with the presence of certain gland cells, and it is hoped that this point can be investigated.

I am indebted to my colleague, Dr. Alan E. Stiven, for useful suggestions and advice relative to the plan of the investigation. The study was further aided by a grant from the Research Council of the University of North Carolina.

SUMMARY

1. At Mount Desert Island, Maine, *Urceolaria spinicola* is of general occurrence on the spines of *Strongylocentrotus droebachiensis*. Two aspects of the urchin-

ciliate relationship were studied, largely on urchins from Frenchman Bay: the occurrence of the ciliate on urchins of different sizes and its distribution on spines of different lengths.

2. The density of the urceolaria population was highest on urchins measuring 24–60 mm. in diameter (test only), assumed to be 2–5 years of age (average number of ciliates per spine, 27). Smaller and therefore younger urchins (diameter, 9–18 mm.) had fewer per spine (average number, 9). On the largest urchins (62–74 mm.), assumed to be at least 6 years of age, urceolarias were extremely scarce (average number per spine, 3). Indeed, many urchins of this size had no ciliates whatsoever.

3. The distribution on spines of different lengths was studied with special care on 41-mm. urchins. The smallest spines (length, 0.6–0.9 mm.) had relatively few urceolarias per spine (average number, 9), whereas spines measuring 1.0–4.9 mm. in length had the largest number per spine (average, 36). The remaining spines (length, 5.0–16.0 mm.) were seriated according to length. On all the sizes, the average number of urceolarias per spine was well below the maximum of 36 and the number decreased as the length of the spine increased. Thus, many of the longest spines lacked ciliates. On spines measuring 0.6 to about 3.0 mm. in length, the urceolarias were distributed uniformly along the length of the spine; on spines longer than 3.0 mm., they were concentrated on the basal half of the spine.

4. The distribution of *U. spinicola* on the spines could not be related convincingly to any of the following factors: degree of ciliation of the spines, contact of the spines with one another, presence of water currents in the environment or availability of bacterial food on the surface of the urchin. Therefore, it is concluded tentatively that the distribution is related to the intrinsic properties of the spine epidermis, perhaps to the distribution of gland cells in it.

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THE GENETICS OF ARTEMIA SALINA. VI. SUMMARY OF MUTATIONS¹

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Artemia salina is of interest to geneticists because some populations are diploid, triploid, tetraploid, or pentaploid (see reviews by Goldschmidt, 1952; Barigozzi, 1957; and Stefani, 1964). Although the cytology of the brine shrimp has been studied for many years, it is only recently that attempts have been made to analyze mutant traits governed by one locus. Cervini (1965) has described the spontaneous recessive autosomal mutation "curly" (*cr*) which causes ventral curling of the abdomen. In our study of gonochoristic diploid populations, we have found seven mutations and many sex mosaics and eye color mosaics. The purpose of this paper is to describe these morphological variations.

MATERIALS AND METHODS

Genetic techniques, glassware, and feeding schedule were described in detail earlier (Bowen, 1962). In brief, two or three nauplii were placed in each vial of culture medium (50 g. NaCl per liter of sea water). Once a week, 0.05 or 0.10 cc. of yeast suspension (1 cc. dry brewers' yeast mixed with 9 cc. of medium) was added to each vial. Inbred stocks are maintained at 21–24° C.; shrimps reach sexual maturity at two to three weeks of age. Origins of the inbred stocks and of the wild populations have been given earlier (Bowen, 1964, 1965).

Macrophotographs of living *Artemia* were taken with a Brinkmann camera (30" bellows) and collimated transmitted light. For histological preparations, shrimps were anesthetized in ether and a few legs were removed to allow entry of fixative; they were placed in Bouin's for 24–48 hours, stored in 70% ethanol, embedded in paraffin, sectioned at 10 μ and stained in haematoxylin and eosin.

India ink was injected into the thorax through micropipettes with tips of 2–4 μ (O.D.) by means of a de Fonbrune micromanipulator. Best results were obtained when shrimps were first anesthetized with ether and then placed within an enclosure improvised from a plastic slide (Dowling, 1963). At the time of injection, the culture medium was drawn off to prevent loss of ink from the micropipette. *Artemia* is able to survive for a few minutes outside the liquid environment.

MORPHOLOGY OF WILD-TYPE ARTEMIA

Fränsemeier (1939), Weisz (1947) and Dutrieu (1960) have described embryonic development. Heath (1924) and Weisz (1946, 1947) have outlined the

¹ Supported by grants from the National Science Foundation (NSF G-13219 and GB-3836). We would like to thank Mrs. Jean Cons who made the histological preparations and Mrs. Jean Chapman who discovered the mosaic shown in Figure 15.

changes during larval development. We have used Heath's diagrams to determine the instar of immature shrimps. The morphology of the adult has been described by Weisz (1947) and Lochhead (1950). The adult body consists of head, 11 thoracic segments, two genital segments, and 6 abdominal segments (Weisz, 1947, p. 81). Each of the 11 thoracic somites bears a pair of phyllopodia (Figs. 2 and 5). The last abdominal somite is fused to the telson which bears the caudal furca (Fig. 5).

The head of *Artemia* bears a pair of slender antennules and a larger pair of antennae. The antennae show sexual dimorphism, being larger and modified for clasping in the male (Figs. 1, 2 and 5).

The median eye consists of three cups, or ocelli. It is red in the first instar and does not gain black pigment until the second (Vaissière, 1961, p. 29). By the third instar, black pigment is usually present in the rudiments of the lateral compound eyes also. The normal compound eye is seen in Figures 7 and 12. The cuticle, which is secreted by the epidermal cells, is not thickened to form a lens. Each ommatidium consists of a cone surrounded by the four crystalline cells which

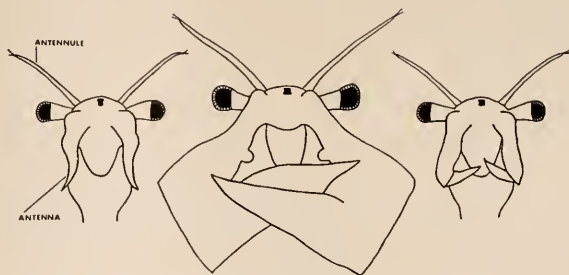
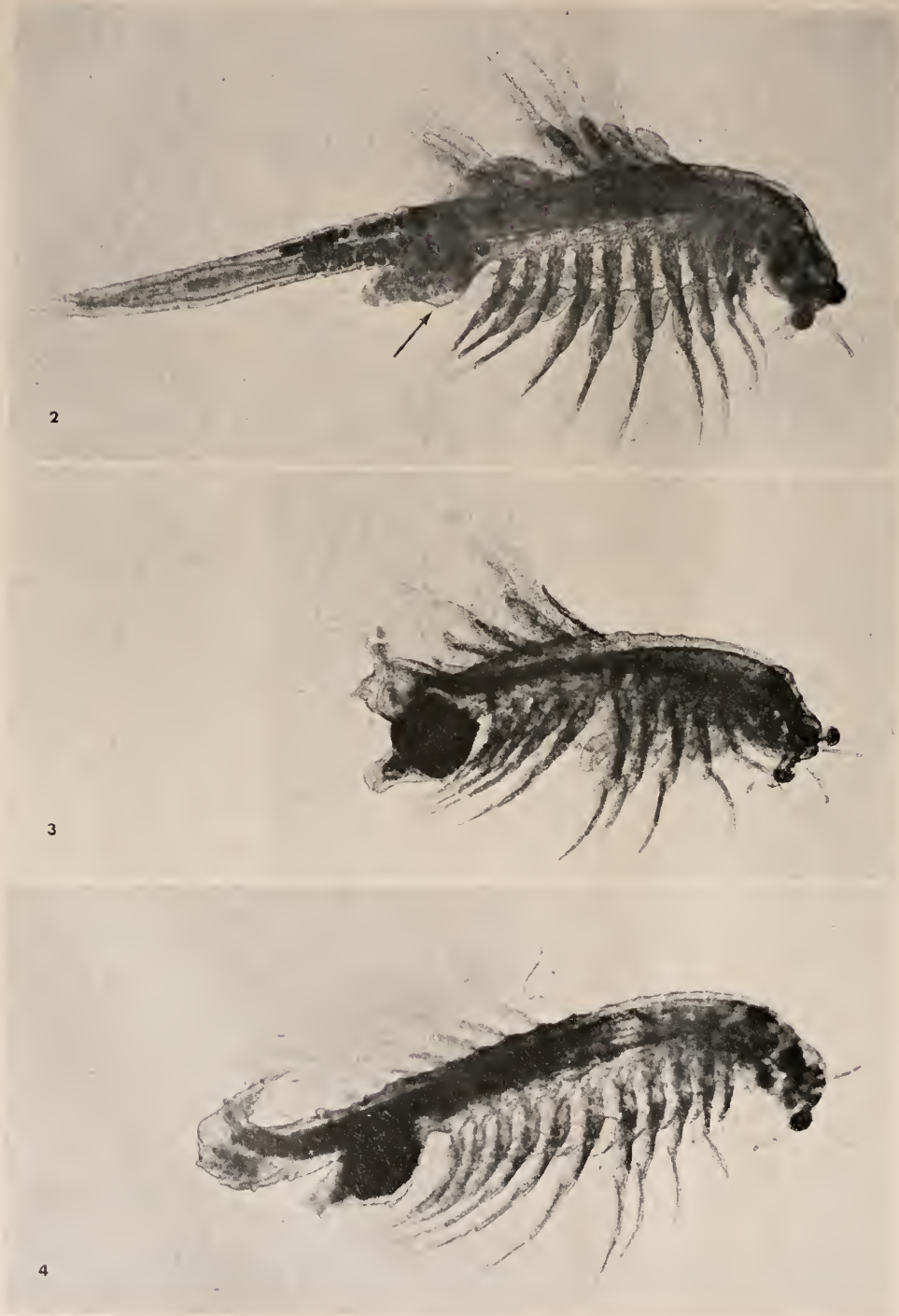


FIGURE 1. Ventral view of head of normal female (left), normal male (center) and *Cu/+* female with curved antennae (right).

secreted it and a proximal rhabdome surrounded by a retinula. The rhabdome lacks the alternating layers of microtubules found in other crustaceans (Eguchi and Waterman, 1965). Each retinula usually contains 5 principal cells and a sixth accessory cell (Debaisieux, 1944, p. 13). The retinular cells contain the photo-stable black-brown pigment which gives the wild-type eye its black color. Each retinular cell is a primary neuron which penetrates the basement membrane and continues as an axon in the fascicular zone of the eyestalk. There are two optic ganglia: the distal lamina ganglionaris and a proximal medulla (Fig. 7). Nerves from the ganglia enter the supra-esophageal ganglion.

The gonads of both sexes are straight cylinders lying above and lateral to the gut in the two genital segments and first few abdominal segments. Gametes leave the anterior ends of the gonads by means of ducts. On each side of the body, the male has a U-shaped seminal vesicle, vas deferens, and penis. The female has two oviducts (lateral pouches) which convey the eggs into a single median uterus wherein they undergo segmentation. Four grape-like clusters of shell glands empty their secretions into the uterus. The oviducts and uterus lie within a ventral median swelling, the ovisac (Fig. 2).



FIGURES 2-4.



FIGURE 5. Dorsal view of living normal male *Artemia*.

FIGURE 6. Dorsal view of cyclops male. The distance between the lines on the right is 0.5 mm.

MORPHOLOGICAL VARIATIONS

A. Variations in morphology of wild populations

Wild-type *Artemia* look very much alike. This is surprising when one considers their geographical isolation: they are found in salt lakes and salterns on

FIGURE 2. Lateral view of living normal female brine shrimp, showing two genital and 6 abdominal segments. The arrow indicates the spine on the ovisac.

FIGURE 3. Lateral view of *s/s* female which has extreme stump expression. Only two genital segments are present.

FIGURE 4. Lateral view of living *s/s* female with moderate stump phenotype.

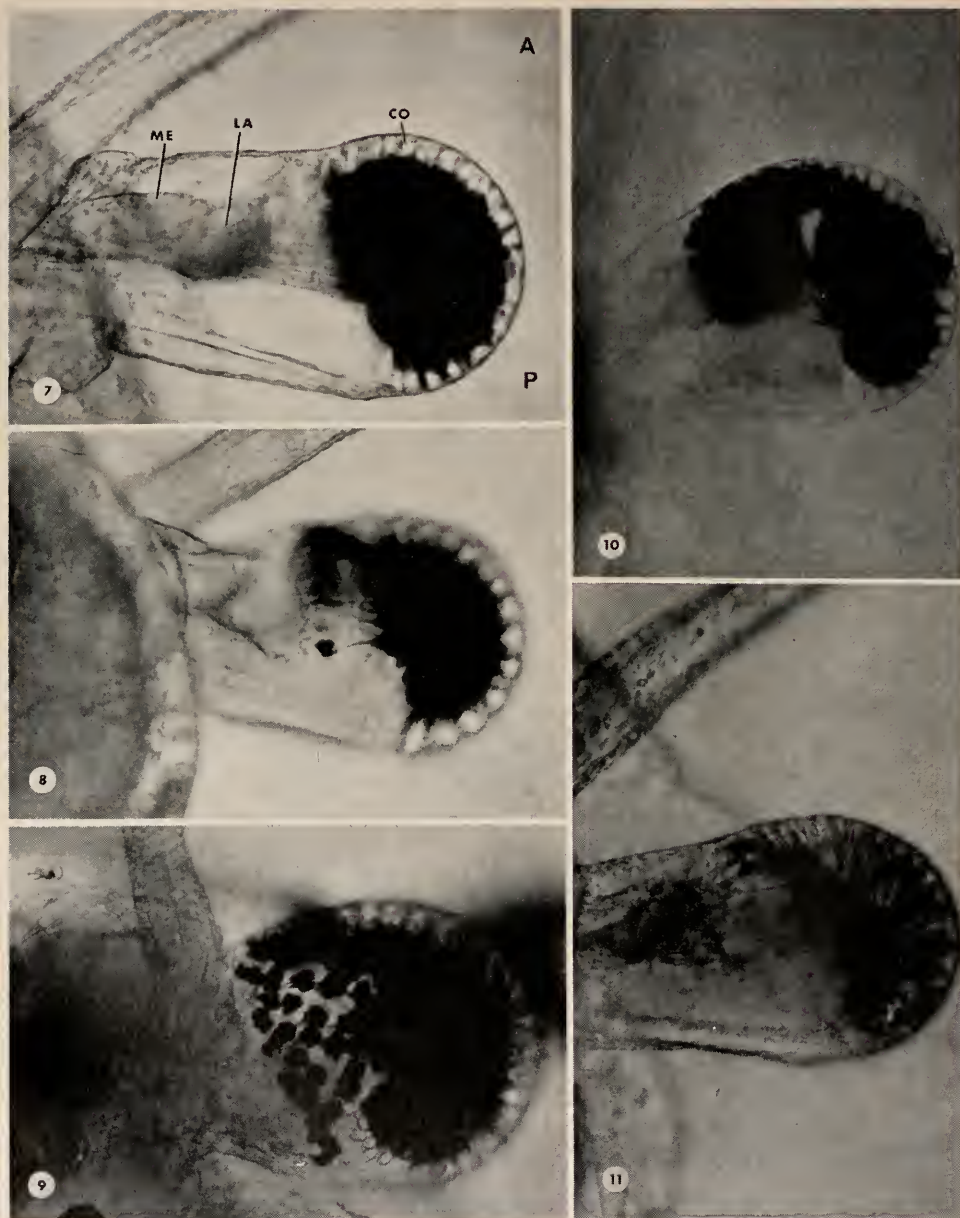


FIGURE 7. Dorsal view of normal compound eye of living brine shrimp. The medulla (ME), lamina ganglionaris (LA) and the cones of the ommatidia (CO) are clearly seen. A, anterior border; P, posterior border of eye. The other photographs on this page are oriented in a similar manner.

FIGURES 8, 9, AND 10. Dorsal views of living shrimps of *c/c* genotype, showing variation of expression of crinkle phenotype.

FIGURE 11. Dorsal view of eye of living garnet shrimp (*g/g* genotype). In areas where the reticular cells have degenerated, the eye is transparent. Few axons remain in the fascicular zone (between lamina and basement membrane).

six continents. Furthermore, certain populations are known to be reproductively isolated. Whereas most American populations are gonochoristic, many European populations are parthenogenetic. American diploid gonochoristic populations are also reproductively isolated from the diploid gonochoristic population from San Bartolomeo near Cagliari, Sardinia (Kuenen, 1939, p. 387; Bowen, 1965). The gonochoristic Mono Lake, California, population is a sibling species which cannot survive in sea water or concentrated brines in which all the other populations thrive (Bowen, 1964).

When reared under identical environmental conditions, some wild-type populations can be distinguished by quantitative differences such as ratio of lengths of abdomen and trunk (see data and review by Gilchrist, 1960). We have examined two parthenogenetic populations (from Sète, France, and from Rottneet Island, near Perth, Australia) and six gonochoristic populations (from Europe, North America and South America) and have detected only a few differences of a qualitative nature. For example, females of the Quemado, New Mexico, population have a small projection on their antennae which is absent in other females (Bowen, 1964). Both males and females from the San Bartolomeo population lack the spikes on the genital segments (seen in Figures 2 and 3) which are present in other populations (Bowen, 1965).

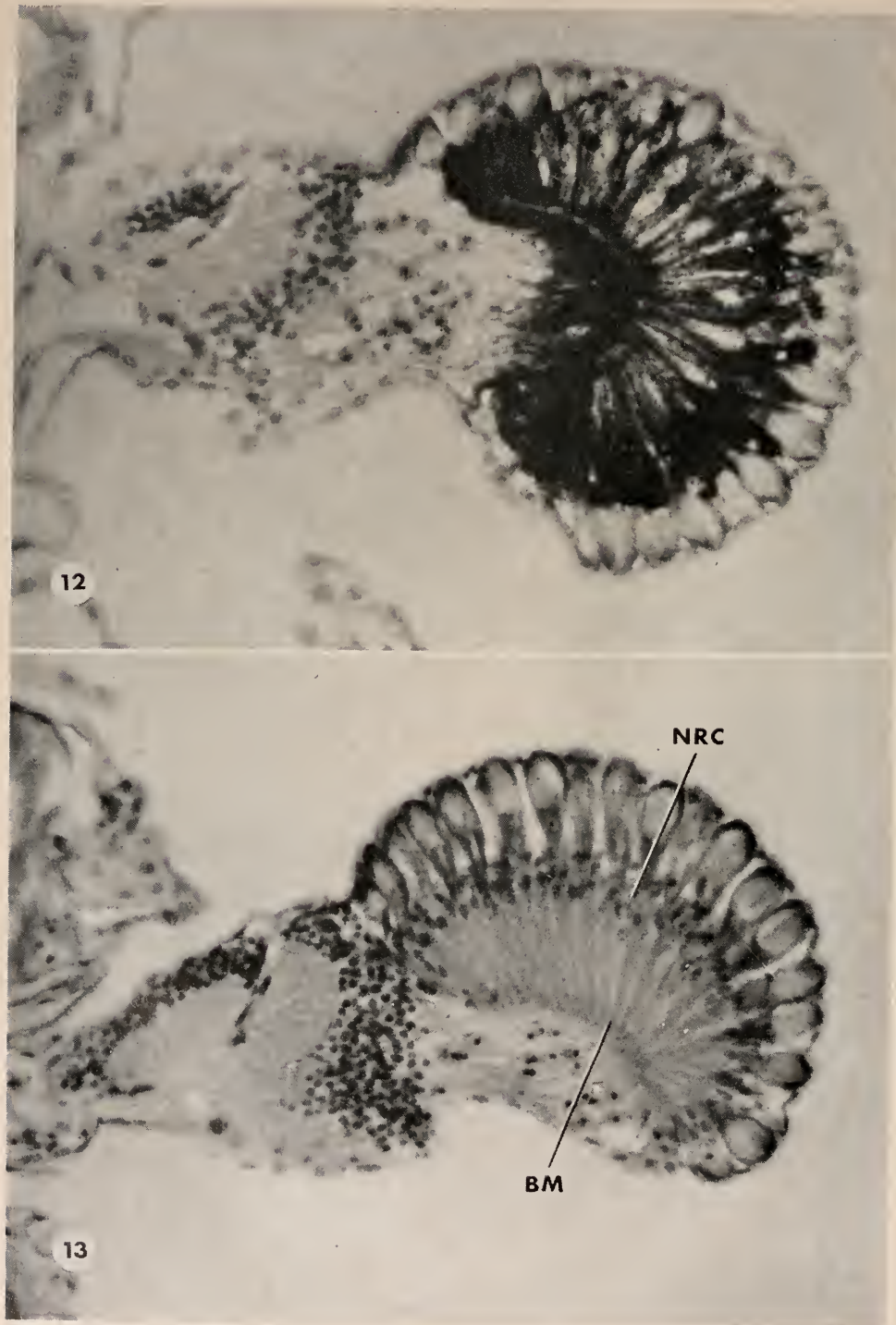
B. *Description of seven mutant genes*

Six of the seven mutations listed below appeared when non-irradiated stocks were inbred by sibling matings; one (garnet) appeared in progeny of x-irradiated shrimps. Two mutations (white and curved) were found by S. T. B.; the other five were discovered by J. H. Five are completely recessive; two (crinkle and curved) have expression in a fraction of the heterozygotes. Six of the seven mutations are carried in our laboratory in pure-breeding cultures; the exception is cyclops which occurs in high frequency in stock #1. Five are autosomal, one (white) is partially sex-linked, and the mode of inheritance of one (cyclops) is not completely known.

1. *Curved (Cu)*. Males homozygous for the mutant gene are normal; expression is therefore said to be sex-limited. Expression in the females is variable, ranging from enlarged, sharply bent antennae (easily seen without a microscope) to antennae which are normal in size but which have a small projection on the posterior surface (visible only when the female is anesthetized and examined under $30\times$ magnification). Females with extreme curved expression have antennae similar to those of normal males (Fig. 1).

The first females with curved antennae were discovered in 1965 among the progeny of a cross between inbred stocks #5 and #12. These females were mated to males from an inbred wild-type stock derived from salterns on Pichilingue Island, Mexico. Of the 313 hybrid female progeny, 56 showed strong expression of curved. In retrospect, it seems probable that the first females were *Cu/+* genotype. The hybrid progeny were inbred for four generations with constant selection of females for strong expression of curved. The result was stock #49 which has high incidence of curved (Table I).

The degree of bending of female antennae increases if animals are reared at



FIGURES 12-13.

27° C. instead of 22° C. At both temperatures, frequency of females with strong expression (detected without use of a compound microscope) increases as the population ages. The *Artemia* described in Table I were reared at 27° C. and classified at an age of five weeks.

Females from the wild-type Pichilingue inbred stock were mated to stock #49 males. Data in the second line of Table I show that 31/52, or 60% of the F₁ females had curved antennae, indicating that this trait is determined by a gene with incomplete dominance which can be transmitted through the male. This excludes the possibility that the curved trait is governed by a gene on the Y chromosome; the female is the heterogametic sex (XY) in *Artemia* (Bowen, 1963a, 1965; Stefani, 1963). The F₁ females were backcrossed to +/+ Pichilingue males. The data in the last line of Table I show that their daughters had curved

TABLE I

*Segregation of gene for curved antennae which has expression only in females
(classification at 5 weeks of age)*

Parental cross		Female progeny classified as curved	
Description	Presumed genotype	Number with strong** expression/total	Number with some*** expression/total
curved stock #49 ♀ ♀ × stock #49 ♂ ♂	Cu/Cu × Cu/Cu	43/46	46/46
non-curved Pich.* ♀ ♀ × stock #49 ♂ ♂	+/+ × Cu/Cu	31/52	41/52
non-curved Pich. ♀ ♀ × ♂ ♂ F ₁ (Pich. ♀ ♀ × #49 ♂)	+/+ × Cu/+	6/22	8/22
curved ♀ ♀ F ₁ (Pich. ♀ ♀ × #49 ♂) × Pich. ♂ ♂	Cu/+ × +/+	12/44	16/44

* Pichilingue inbred wild-type stock.

** Strong expression indicates that unanesthetized females were classified as curved after observation under a dissecting microscope (7 ×).

*** Some curved expression includes those with strong expression and those with mild expression (seen only on anesthetized females under 30 ×).

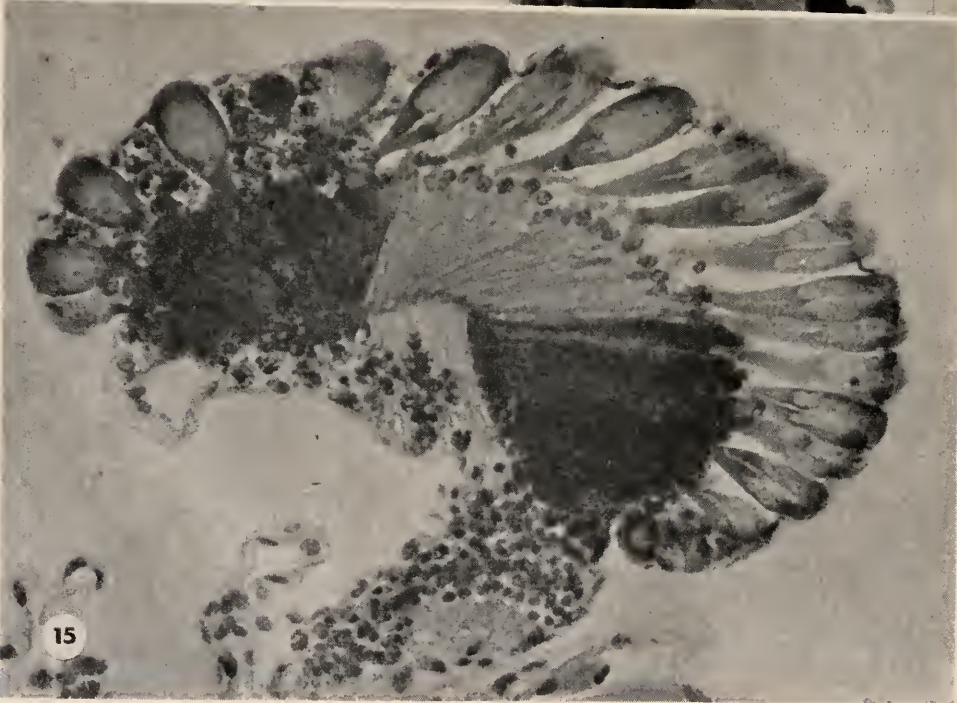
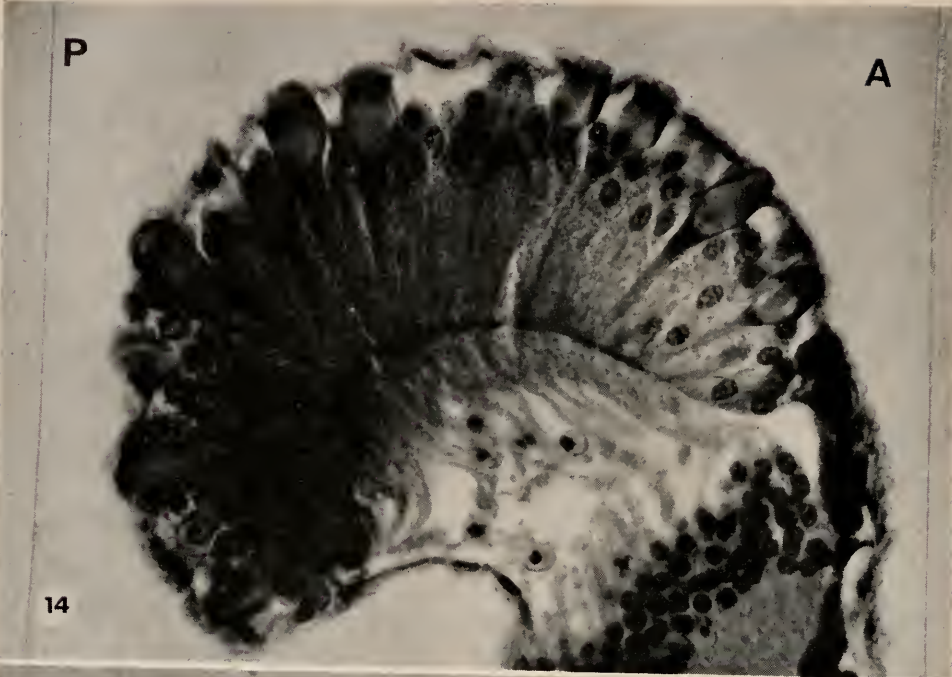
antennae. This demonstrates that curved is not located on the X chromosome. (If curved were on the X, the F₁ females would be X^{cu}Y⁺ and would be unable to transmit the mutant gene to their female offspring.)

We conclude that curved is a dominant autosomal sex-limited gene with incomplete penetrance and variable expression. Females with mild expression have a projection of the posterior surface of their antennae, as do wild-type Quemado females.

2. *Stump* (*s*). This autosomal recessive mutation was discovered in 1960 during inbreeding of wild-type shrimps from salterns on San Francisco Bay. In some *s/s* shrimps, the abdomen is normal. In others, it is twisted dorsally (Fig. 4), or it lacks from one to six segments. The female shown in Figure 3 lacked all

FIGURE 12. Longitudinal section of normal compound eye. Black pigment is within the reticular cells.

FIGURE 13. Longitudinal section of eye of shrimp with *w/w* (white) genotype. The basal membrane (BM) and the nuclei of the reticular cells (NRC) are seen. The reticular cells contain opaque white pigment. All histological preparations (Figs. 12–17) were prepared in the same manner (haematoxylin and eosin).



FIGURES 14-15.

six abdominal segments. Although her second genital segment was attached directly to the telson, she had normal fertility.

Matings of stump males to stump females gave rise to a stock of *s/s* shrimps in which only 37% of the shrimps showed sufficient expression to be classified as stump (when viewed under a $7\times$ dissecting microscope). From matings within this *s/s* stock, the ratio of stump to non-stump progeny was the same when extreme stump parents were selected as when non-stump parents were selected. This suggests that the *s* gene has low penetrance in homozygotes.

3. *Red (r)*. This autosomal recessive mutation appeared during inbreeding of a stock from Great Salt Lake, Utah, in 1960. Segregation data and descriptions of *r/r*, *r/+* and *+/+* shrimps appeared earlier (Bowen, 1962). Briefly, *r/r* shrimps have colorless compound eyes from the third to the fifth instar. Then deposition of red pigment begins in the posterior border and rapidly progresses anteriorly (Fig. 18). Median eye and compound eyes are bright red from the seventh through the thirteenth instars (about two to three weeks of age). Shortly after sexual maturity, brown-black pigment appears in the caudal reticular cells of the compound eyes. Deposition of black pigment also progresses anteriorly, masking the red pigment within 48 hours (Fig. 14). The median eye may also darken, but more often remains red. A similar mutation governs rate of eye pigment production in the amphipod, *Gammarus chevreuxi*. The gene *d* (delayed melanin) delayed deposition of pigment until the amphipods were sexually mature (Ford and Huxley, 1929).

4. *Cyclops (cy)*. During development of cyclopean metanauplii, the lateral eyes give the illusion of moving forward and fusing together in the midline as a single large compound eye (Figs. 6 and 20). The eyes are in the normal location at the fourth instar; fusion is complete by the ninth instar. Histological preparations indicate that ganglia and nerves of the two optic stalks fuse. The eye of the cyclopean *Artemia* is similar to the normal eye of the cladoceran, *Leptodora*.

Cyclopean *Artemia* occur sporadically in stock #1 (*r/r* genotype) which is descended from the Great Salt Lake population. Nine cyclops were observed in this stock in 1961. Two died before sexual maturity. Of those that matured, four were male and three were female. Only two produced offspring. In the first successful mating, a cyclopean male (*r/r*) was outcrossed to a wild-type female. Of the 27 progeny, only 9 lived to maturity; all were non-cyclopean *r/+* shrimps. These were bred *inter se* but none of the 255 F_2 progeny was cyclopean. In the second successful mating, a cyclopean female was mated to her brother and produced 42 nauplii, of which 11 reached maturity. One was a male cyclops which failed to produce progeny. His sibs were mated *inter se*; of 140 offspring, 75 reached maturity and all had *r/r*, non-cyclopean eyes. This finding is quite different from the results obtained when the sibs of another cyclops were mated: three of the 51 progeny were cyclopean.

FIGURE 14. Longitudinal section through eye of a sexually mature shrimp of *r/r* genotype. A, anterior; P, posterior. Deposition of black pigment has begun in reticular cells in the posterior part of the eye. The anterior portion is still bright red.

FIGURE 15. Section through eye of sex mosaic #14 which was also mosaic for eye color. There is a central patch of white tissue surrounded by pigmented tissue of *r/r* genotype (black pigment in this mature shrimp). Note that pigmented and white reticular cells lie side by side with no areas of intermediate color.

The study of cyclops was abandoned because the cyclopean shrimps had low viability and fertility. The trait may be governed by a recessive gene which has low viability or low penetrance, or it may be affected by more than one locus. A future study might be made of stock #1 in varying ionic environments or at different temperatures, in an attempt to increase the frequency of cyclopean shrimps.

5. *Crinkle (c)*. In immature shrimps with c/c genotype, the compound eyes are normal. After sexual maturity, some reticular cells detach from the ommatidia with the result that the eye becomes mottled in appearance. In c/c shrimps six weeks old or older, the distal ends of some reticular cells lie in the eye stalk rather than in the normal eye field. A characteristic "crinkle patch" (containing reticular cells but lacking cones) appears in the anterior dorsal region of the stalk, medial to the basement membrane (Figs. 8, 9 and 10).

The first crinkle-eyed shrimp appeared in 1960 in an inbred stock derived from salterns on San Francisco Bay (Bowen, 1963a). From a backcross of $c/+$ to c/c shrimps, 116/274, or 42% of the progeny had crinkle phenotype at the age of five weeks. The frequency had increased to 114/224, or 51%, when these backcross progeny reached 7 weeks of age, because expression of c/c genotype becomes

TABLE II
Segregation of the gene g

Type of mating	Classification of progeny (at 4 weeks of age)		Total
	Wild	Garnet	
$g/g \times g/g$	0	301	301
$\sigma^+ +/+ \times \text{♀ } g/g$	105	0	105
$\sigma^+ g/g \times \text{♀ } +/+$	297	0	297
$\sigma^+ +/g \times \text{♀ } g/g$	158	133	291
$\sigma^+ g/g \times \text{♀ } +/g$	261	240	501

more pronounced with age. Unfortunately, the crinkle gene has some expression in a small fraction of heterozygotes and this frequency is also increased with age. Evidence for this is seen in data from a heterozygous F_1 population. At four weeks of age, 3/90 $c/+$ shrimps had crinkle eyes; at 10 weeks of age, 8/53 were crinkle-eyed. The frequency of expression in $c/+$ heterozygotes may vary with environment and genetic background as well as with age.

6. *Garnet (g)*. This mutant eye color first appeared in 1961 in the F_2 of two shrimps from San Francisco cysts which had received 10 kr of x-irradiation (Bowen, 1963b). After a pure-breeding stock was established, reciprocal crosses were made between garnet and wild-type and testcrosses were made of the F_1 . The data in Table II indicate that garnet eye color is due to an autosomal recessive gene which has complete penetrance in the homozygote and no expression in the heterozygote.

Eye color becomes progressively lighter as the g/g shrimp ages. In the first three instars, the eyes cannot be distinguished from wild-type. However, at one week of age (fourth to seventh instar), the eyes become dark brown or red-brown (garnet). This mutation affects both eye color and structure. At sexual maturity

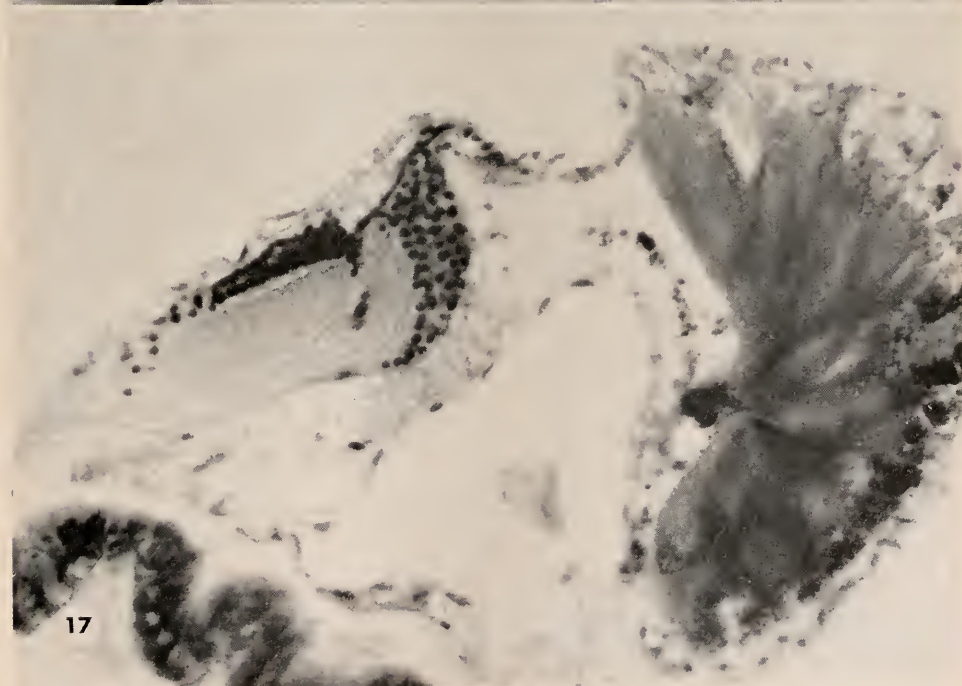
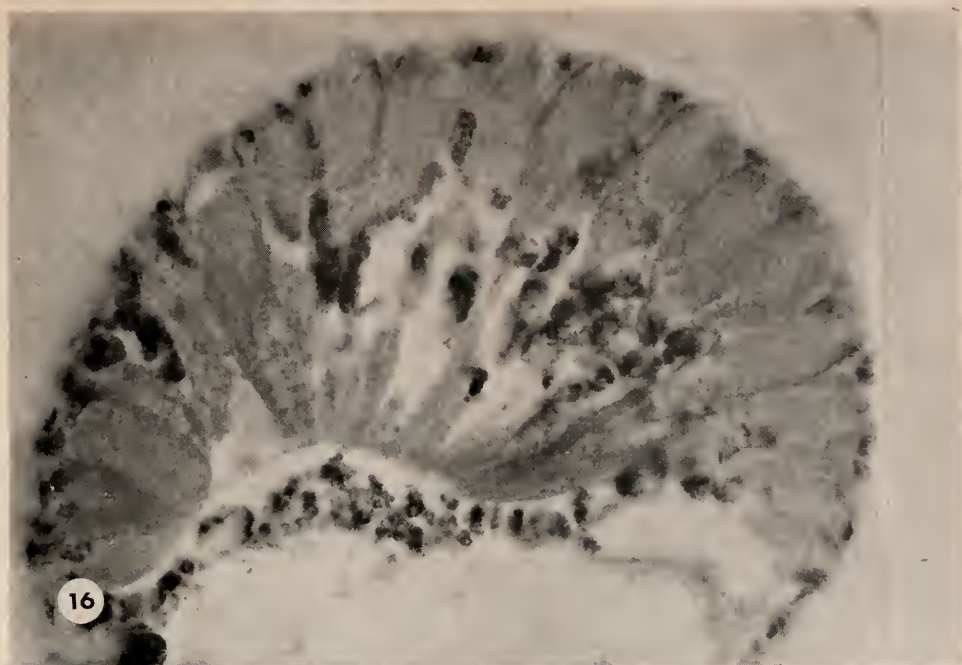
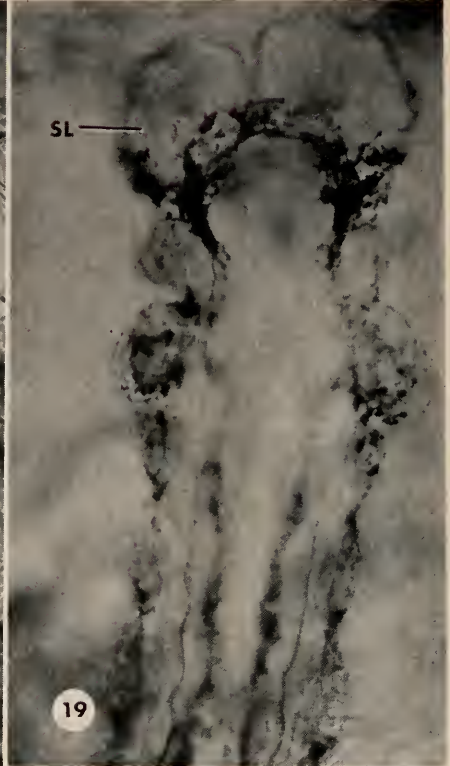


FIGURE 16. Longitudinal section through tip of the eye of a garnet (*g/g*) shrimp. Whereas the rhabdome remains intact, the reticular cells have degenerated, leaving cell fragments filled with garnet pigment above and below the basement membrane.

FIGURE 17. Longitudinal section through eyestalk of a garnet-eyed shrimp. The reticular cell axons, which normally lie between the basement membrane and the distal optic ganglion, have degenerated.



FIGURES 18-21.

(three weeks of age), the garnet eyes often have an irregular proximal border. Many retinular cells degenerate, causing the eye to be flecked with clear areas. By 6 to 8 weeks of age, the compound eyes of *g/g* shrimps have irregular patches of garnet pigment only at the periphery of the eye; some eyes are almost colorless (Fig. 11). The median eye is often unpigmented also. In histological preparations, it is seen that retinular cells detach from the rhabdome and basement membrane. Many disintegrate. Those that remain are small spherical cells (or cell remnants) which lie medial to the basement membrane or in the periphery of the eye field (Fig. 16). Note that the rhabdome remains structurally intact after degeneration of the retinular cells. At the age of two months, no axons can be seen in the zone between basement membrane and distal ganglion (Fig. 17). By this time, garnet-eyed shrimps have lost the tendency to orient with their ventral surface toward a light source.

Correlated with a decreasing pigmentation of the retinular cells is an increasing deposition of garnet pigment in other specialized cells. By the sixth instar, garnet pigment is seen in the antennal glands. By the eighth instar, pigment is present in the phagocytic storage cells and in the maxillary glands. When *g/g* shrimps reach four weeks of age or more, they have conspicuous pigmented areas around the caudal walls of the lobes of the stomach, on the lateral surface of the anterior part of the digestive tract, and on the outside of the anterior portion of the heart. These are sites where phagocytic storage cells are concentrated. To demonstrate this, wild-type shrimps were injected with India ink. Ink was found in cells in the phyllopodia, in the maxillary glands, and scattered along the outer walls of the heart. These phagocytic cells were particularly dense on the outside of the lateral walls of the gut in the anterior thorax. In Figure 19, the characteristic "inverted U" distribution of ink-filled phagocytic cells across the dorsal surface of the stomach walls is seen. The garnet pigment in mature *g/g* shrimps has an identical distribution.

In order to determine the mechanisms of cellular degeneration and of pigment dissolution, a study should be made of the ultrastructure of the eye in *g/g* shrimps, with particular attention to changes in the lysosomes with age. It is interesting to note that a recessive mutation (albino) which brought about complete degeneration in retinular cells appeared in the amphipod *Gammarus chevreuxi* (reviewed by Sexton and Clark, 1936, p. 365).

7. *White (w)*. The gene for white eyes is recessive and partially sex-linked; that is, the white locus is on the homologous segment of the sex chromosomes

FIGURE 18. Ventral view of living sixth instar metanauplius of *r/r* genotype. The median eye is red. The anterior portions of the two compound eyes lack pigment. Red pigment is being laid down in posterior ommatidia; this process will proceed anteriorly until the entire eye becomes red.

FIGURE 19. Dorsal view of head and upper thorax of living wild-type female which has been injected with India ink. Note ink-filled phagocytic cells concentrated in a U-shaped area above the posterior walls of the stomach lobes (SL).

FIGURE 20. Dorsal view of eye of living cyclops male.

FIGURE 21. Lateral view of genital segment of living mosaic male #3 which has one "penis" which is a mixture of ovisac and penis structures proximally and phyllopodium structures distally. The arrow indicates the spine of the "ovisac"; SV, seminal vesicle filled with sperm.

(Bowen, 1963a). In matings of X_wX_w males to X_wY_+ females or to X_+Y_w females, crossing over can be detected. The amount of recombination between the white locus and the sex locus varies from 0.05% to 20%, depending upon which female line is tested. The characteristic crossover frequency is transmitted matroclinously (Bowen, 1965).

The gene w has no expression in the heterozygote and has complete penetrance in the homozygote. The lateral and median eyes in w/w shrimps are white throughout the lifespan with one complication: some w/w shrimps develop a pink or bright orange cast to their eyes. Orange pigment may be in the retinular cells and/or in the nerve and ganglia in the eye stalk. Attempts to select for orange color in breeding experiments have failed. Further evidence that this trait is not heritable is the fact that if w/w shrimps with orange eye color are transferred to fresh culture medium, the color will fade within a few weeks, which suggests that the orange tinge must be due to the storage of some material obtained from the food.

If one compares white eyes and garnet eyes (Figs. 13 and 16), one sees that aging garnet eyes become transparent due to degeneration of retinular cells, whereas white eyes contain opaque white pigment in their retinular cells.

C. Gene interactions (r , g and c) and linkage

Shrimps with c/c ; g/g genotype have reddish-brown eyes as they approach sexual maturity. Three weeks later, crinkle patches on the eye stalk appear but they are difficult to see because at this time the retinular cells degenerate under control of the garnet gene. Shrimps with c/c ; r/r genotype have dark red eyes as they approach sexual maturity. Three weeks later, the main eye fields turn black, but the crinkle patches remain red.

The most useful genetic marker is the mutation for white eyes. It has complete penetrance in the homozygote, no expression in the heterozygote, and is easily classified at all ages and in all environments. The other mutations fail to meet one or more of these criteria. For this reason and also because of complex interactions between the mutations affecting the eyes, linkage tests are tedious to carry out. At the present time, the only linkage relationship which has been established is that between the white locus and the sex locus (Bowen, 1965).

D. Epistasis and tests for allelism (eye color genes: r , g and w)

In order to determine if the three eye color mutations were allelic, the following stocks were crossed: garnet $\times$ red, garnet $\times$ white, and red $\times$ white. Because the F_1 progeny from the three crosses were wild-type, we conclude that the three mutations are not alleles.

The gene w , when homozygous, is epistatic to the gene r . Stock #10 breeds true for white-eyed males (X_wX_w ; r/r) and red-eyed females (X_wY_+ ; r/r) because crossing over between the X and Y is suppressed in this stock.

White is also epistatic to garnet. Evidence for this is seen in the results of a cross of garnet-eyed shrimps: g/g ; X_+Y_w females to g/g ; X_+X_w males. Twenty-seven per cent (57/211) of the progeny were white-eyed. Of the 57 white-eyed shrimps, 53 were females and 4 were males resulting from crossing over.

E. Discussion of the mechanism of gene action (r , g and w)

The black eye pigment of wild-type *Artemia* is an ommochrome (Becker, 1942). Probably the gene for garnet eyes affects ommochrome degradation rather than ommochrome synthesis for two reasons: (1) reticular cells of young g/g shrimps contain normal black pigment, and (2) reticular cells of sexually mature g/g shrimps degenerate as a result of the action of the garnet gene.

We will discuss three alternative hypotheses for the mode of action of the gene for white eye color:

1. The gene w may act on the stroma of the eye pigment granule, either by causing a complete absence of the stroma or by producing a defect in its structure. This hypothesis would account for the fact that w/w ; r/r and w/w ; g/g shrimps have white eye color. The white eyes should be examined with the electron microscope to determine if pigment granules are absent or changed in structure. Nolte (1961) has reported a great reduction in the number of granules in the retinulae of st/st and v/v *Drosophila* which lack ommochrome pigment.

2. The gene w may alter reticular cell membrane permeability in such a way as to prevent the entrance of ommochrome precursors into the cell. This hypothesis would also account for the fact that the gene for white is epistatic to the genes for red and garnet.

3. (a) The genes for white and for red eyes may be changes in structural genes which code enzymes in the biosynthetic pathway of ommochrome eye pigment. Because white is epistatic to red, the enzyme controlled by the white locus would act earlier in the ommochrome synthetic pathway than the enzyme controlled by the red locus. (b) Along the same line of reasoning, it is possible that w and r may be changes at operator or repressor loci which indirectly control enzymes in ommochrome synthesis. Some doubts about hypothesis #3 have been raised by the discovery of mosaic shrimps with compound eyes which contain black and white reticular cells lying next to one another (described under section G of this paper). If the white cells lacked an ommochrome precursor, one might expect it to enter the white cells by diffusion from adjacent normally pigmented cells, resulting in a gradient of white, intermediate, and normal cells. Because no intermediate cells were seen, we conclude that if hypothesis #3 is valid, the gene w^+ must govern an enzyme which catalyzes the synthesis of a non-diffusible precursor of the ommochrome eye pigment.

E. Modifications of unknown origin

In a study of the effects of x-irradiation upon encysted blastulae (Bowen, 1963b) variants were discovered among the inbred descendants of shrimps in the 2-kr and 10-kr x-irradiation treatments. There were five independent occurrences of absence of setae on the exopodites and three occurrences of swollen abdomen. Other variants were: bent abdomen, kidney-shaped eyes, swollen branchiae, and a ventral median projection on the fifth segment of the abdomen. The only viable pure-breeding stock that could be developed from the progeny of x-irradiated shrimps was the garnet-eye stock.

Many variants occurred in non-irradiated stocks. One male had no antennae

whatsoever. Several animals in one r/r line showed deposition of the red pigment delayed until sexual maturity. Many shrimps in a wild-type stock lacked a median eye. There were five independent occurrences of shortened, twisted abdomens (in addition to stump, described above). These morphological variants could not be developed into mutant stocks for one of these reasons: the shrimps died without progeny, sib matings gave all wild-type F_2 progeny, the traits had low viability or penetrance, or the stock lost vigor with inbreeding.

F. *Mosaics*

Eighteen mosaics are described in Tables III and IV. Eleven were sex mosaics, three were mosaics combining genitalia and phyllopodia tissue, one was a female with abnormal legs on one side, one was a male lacking an antenna, and two were metanauplii which had eyes of unequal sizes. The last two hatched from cysts given a lethal dose of 50,000 r x-irradiation and died before maturity (Bowen, 1963b).

The three phyllopodia-genitalia mosaics (mosaics 1, 2 and 3 in Table III) suggested that structures within the phyllopodia are homologous with those in the genital segments. Mosaic #3 is a white-eyed male descended from a cross between a black-eyed mother (X_wY_+) and a white-eyed father (X_wX_w) from stock #9. On each side of the body, there is a normal testis filled with sperm and a male antenna. On the right side, the last four thoracic appendages are shortened and deformed. On the left side of the genital segments, there is a normal penis containing a vas and seminal vesicle. On the right, the external genitalia are a mixture of ovisac and penis structures proximally; distally, the structure becomes a phyllopodium (Fig. 21).

Of the 11 sex mosaics (numbers 8 to 18, in Tables III and IV), three were perfect bilateral gynandromorphs. Each had a testis containing sperm on one side and an ovary producing yolky eggs on the other. One has been described in detail earlier (Bowen and Hanson, 1962). Another sex mosaic (number 11 in Table III) consisted of male tissue except for the presence of shell glands filled with brown secretion. Mosaic No. 18 (Table IV) consisted of female tissue except for one perfect male antenna. The presence of a small amount of tissue characteristic of one sex in an animal composed for the most part of cells of the other sex is usually interpreted as evidence that sex is determined autonomously. We can be certain that if a sex hormone is present in *Artemia*, it does not suppress the differentiation of cells with the chromosome constitution of the opposite sex. In each of the 11 sex mosaics, the internal organs were male or female rather than intersexual in character. The external shape of the antennae and genitalia were sometimes intermediate, but this could be attributed to mixtures of cells with male or female genotypes in the epidermis. Therefore, all the mosaics were in accord with the hypothesis that each cell is either male or female in phenotype.

Although we know that female *Artemia* are XY and males are XX, we do not know whether the female phenotype is due to (1) female-determining gene(s) on the Y or to (2) the balance of the two sets of autosomes (bearing genes for femaleness) to the single X chromosome (bearing genes for maleness). Each of the five sex mosaics in Table IV resulted from the cross of a white male from stock #9 to a $w/+$ female. It was hoped that some insight into sex determination

TABLE III
Descriptions of thirteen mosaic shrimps

Mosaic no.	Code*	Description
1	s	Normal male antennae and male reproductive organs in genital segments. Five appendages on left side of thorax are mixtures of male and female genitalia.
2	s	Both testes filled with sperm, normal male antennae. External genitalia on both sides are a mixture of penis, ovisac, and phyllopod structures. Vas is continuous with internal structures of phyllopod.
3	s	On both sides, there is a normal male antenna and a testis filled with sperm. <i>Left side:</i> normal penis containing vas and seminal vesicle. <i>Right side:</i> the genitalia are a mixture of ovisac and penis structures proximally; distally there is a phyllopodium (Fig. 21).
4	s	Female with normal antennae, ovaries, and genitalia. On two thoracic segments, one leg is shortened, another slants dorsally.
5	px	Normal male genitalia and testis on both sides. One antenna has normal male shape; other is a broad stump.
6, 7	x	Two metanauplii; each has eyes of disparate size.
8, 9, 10	px	Three perfect bilateral gynandromorphs.
11	px	<i>Right side:</i> male antenna, testis filled with sperm, male genitalia (although penis lacks external spine). <i>Left side:</i> antenna is intermediate in shape and lacks frontal knob. Testis is filled with sperm. External genitalia are mixture of ovisac and penis structures; within lie seminal vesicle, vas, and shell glands (attached to vas deferens).
12	px	<i>Right side:</i> small lump in place of antenna, testis filled with sperm. Although penis is normal, seminal vesicle is straight and vas is missing. <i>Left side:</i> normal male antenna, testis filled with sperm; external genitalia are a mixture of ovisac and penis structures. A short spherical seminal vesicle is filled with sperm.
13	s	Perfect female antenna on left, intermediate antenna on right lacks frontal knob. Normal ovisac. In this immature specimen, the sex of the gonad and accessory organs could not be determined.

* x, mosaic hatched from x-irradiated cyst (50,000 r);

px, mosaic found in progeny of x-irradiated shrimps (2,000 or 10,000 r);

s, spontaneous occurrence in non-irradiated stocks.

could be gained from study of these mosaics whose sex chromosomes carry eye color markers. However, no conclusions can be drawn because each of the mosaics in Table IV could have resulted from (1) a normal zygote with somatic non-disjunction occurring in an early cleavage division, (2) a binuclear oocyte with both nuclei fertilized, or (3) a binuclear oocyte with only one nucleus fertilized. Goldschmidt (1952, p. 123) reported finding binuclear oocytes in California *Artemia*. This problem is further complicated by crossing over between the X and Y; an estimate of crossover frequency in the five mothers can be obtained from the third vertical column in Table IV.

Histological examination of mottled eyes (in mosaics #17 and 18) revealed that retinular cells containing white pigment were adjacent to retinular cells with wild-type black pigment. In mosaic #14, white ommatidia were adjacent to red ommatidia which turned black as the animal aged. There were no intermediate cells bearing a reduced amount of dark pigment (see Fig. 15). This indicates that there is no diffusible substance produced by wild-type or by *r/r* retinulae which is lacking in white retinulae; that is, the type of pigment is determined

TABLE IV

*Five mosaic progeny of non-irradiated parents
(w/w fathers and w/+ mothers)*

Mosaic no.	Parents*		Siblings and progeny of similar matings	Description of mosaic	
	Mother	Father		Left side	Right side
14	$X_w^9 Y_+^6$	$X_w^9 X_w^9$	1 pigm. ♂	Red median eye	
			2 white ♀ ♀ 513 pigm. ♀ ♀ 379 white ♂ ♂	Some white reticular cells surrounded by r/r cells (red, changing to black at maturity). Normal male antenna.	White compound eye. Proximal segment of antenna male; distal part female.
				Male gonad, genitalia, and sperm present on both sides.	
15	$X_w^9 Y_+^9$	$X_w^9 X_w^9$	623 white ♂ ♂	Red median eye	
			877 pigm. ♀ ♀	Red compound eyes	
				Normal male antenna. Shell glands present. Mixed male and female genitalia.	Normal female antenna. Female ovisac and shell glands.
16	$X_w^9 Y_+^Q$	$X_w^9 X_w^9$	69 pigm. ♀ ♀ 59 white ♂ ♂ 8 white ♀ ♀ 15 pigm. ♂ ♂	White compound eye. Larger antenna, mixed male and female characteristics. Female gonad, yolky eggs. Genitalia mixed.	Black compound eye. Smaller antenna, mixed male and female characteristics. Female gonad, yolky eggs. Female genitalia; oviduct, 2 shell glands, half-uterus.
17	$X_+^{SF} Y_w^{11}$	$X_w^9 X_w^9$	264 white ♀ ♀ 294 pigm. ♂ ♂	Black and white reticular cells in eye.	White compound eye.
			31 white ♂ ♂ 36 white ♀ ♀	Normal female antennae, gonads and genitalia on both sides of body.	
18	$X_w^9 Y_+^{SF}$	$X_w^9 X_w^9$	149 white ♂ ♂	White and black (mottled) median eye	
			149 pigm. ♀ ♀ 8 white ♀ ♀	Black compound eye. Female antenna.	White compound eye. Male antenna.
			6 pigm. ♂ ♂	Male gonad, genitalia and sperm present on both sides.	

* In each genotype, subscripts indicate the allele on the sex chromosome; superscripts designate origin of the differential segment of the sex chromosome (inbred stocks 5, 9, 11; and wild populations: SF, San Francisco Bay; Q, Quemado, New Mexico).

autonomously by the genes rather than through the mediation of hormones or other diffusible substances.

SUMMARY

1. Seven mutant genes of the brine shrimp have been studied. The mutation *s*, stump, shortens the abdomen; in extreme cases, all six abdominal segments are missing. An autosomal sex-limited mutation, *Cu*, curved, determines that females will have small curved antennae similar to those of the male. Two mutations (*w*, white and *r*, red) affect color of the eye and two mutations alter eye structure (*cy*, cyclops and *c*, crinkle). The garnet mutation, *g*, affects both color and structure of the eye.

2. Five of the mutant genes are autosomal, one (white) is partially sex-linked, and the mode of inheritance of one (cyclops) is not completely known.

3. Injections of India ink were used to demonstrate the distribution of phagocytic cells. These cells also take up pigment released by degenerating retinular cells in garnet-eyed shrimps.

4. The 11 sex mosaics are consistent with the hypothesis that each cell is male or female rather than intersexual in character.

5. Four shrimps had eyes which were mosaic for red and white or for black and white retinular cells. This suggests that eye pigment is determined autonomously; that is, there is no diffusible factor produced by red or wild-type retinular cells which is lacking in white cells.

6. The gene for white eyes, when homozygous, is epistatic to the genes for garnet and for red eyes. Three possible modes of action of the gene *w* are discussed.

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MOTILITY AND AGING OF ARBACIA SPERM¹

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It is well known that diluted sea urchin sperm have a relatively short effective life span. In a few hours or days they lose the ability to activate eggs, become immotile and their respiration ceases (Gemmill, 1900; Cohn, 1918; Gray, 1928, 1931; Rothschild, 1951; Tyler, 1953; Rothschild and Tyler, 1954; Bishop, 1962; Mann, 1964).

The loss of vitality of sperm is reported to be associated with the exhaustion of energy reserves (Gemmill, 1900; Cohn, 1918; Tyler, 1953). Gemmill recognized that sperm were more active, and also pointed out (p. 171) that "on comparing the movement of spermatozoa in different mixtures [dilutions], one finds that the difference in activity is not sufficiently marked to account for the very early loss of vitality of spermatozoa in the weaker mixtures simply in terms of exhaustion of energy." The relationship between the motility of sea urchin sperm and the rate at which they lose the ability to activate eggs remains somewhat uncertain, primarily because it is difficult to evaluate quantitatively the motility of spermatozoa.

The problems of finding the motility status of semen samples are manifold. Microscopic examination to determine sperm activity involves many variables that are difficult to control (Bishop, 1962; Rikmenspoel, 1962; Rothschild, 1953; van Duijn, 1963, 1964). The impedance change frequency method for rating motility (Rothschild 1948a) allows better control of these variables but is unsuitable with diluted semen. A simple method of rating sperm motility which avoids some of these problems was devised for this study.

The first part of this report deals with an analysis of the method for rating motility. The second part is an investigation of senescence of sperm, utilizing the method for rating motility. The following results show that under the conditions of these experiments the concentration, motility and fertility of sperm suspensions diminished most rapidly soon after dilution and more slowly later on. The rapid initial decline in fertility and concentration was prevented by experimentally immobilizing the spermatozoa, but immotile semen ultimately lost fertility at about the same time as motile semen.

MATERIAL AND METHODS

Arbacia punctulata was furnished by the Supply Department of the Marine Biological Laboratory at Woods Hole, Mass. Gametes were obtained by electrical stimulation of the intact animals (cf. Costello *et al.*, 1957). Sperm were collected

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in 100- or 250-ml. beakers of sea water by immersing the aboral surface of the urchin, along with one electrode of the stimulator, and applying the other electrode to the oral surface. Sperm accumulated in piles on the bottom of the beaker in a relatively undiluted state. The time of the first stirring of the sperm into suspension was considered the beginning of the experiment. Eggs were shed in a similar fashion into 100 ml. of sea water, but only a few thousand at a time so that the same female could be used repeatedly to produce fresh eggs.

Sperm concentration was determined by counting in a Neubauer hemocytometer and/or by measuring light absorption with a Klett-Summerson colorimeter (green filter, *cf.* Iverson, 1964).

Fertilizing capacity was determined by diluting sperm in two-fold steps, then adding about 500 eggs to each dilution (total volume 5 ml.). Fertilizing capacity was rated numerically by taking the reciprocal of the sperm concentration at which less than 100% cleavage was attained and above which all eggs were fertilized.

Motility was rated by comparing the sedimentation of living and formalin-killed portions of sperm suspensions. Sedimentation was enhanced by motility. In order to rate motility experimentally, two 15-ml. portions of diluted sperm suspensions (usually about 20 million cells/ml.) were withdrawn and one portion killed with 0.02% formalin. Sperm concentration of the two portions was determined by optical density (O.D.) measurements. Both samples were then centrifuged at 200 *g*, 20° C. for 20 minutes in conical tubes in swinging buckets. After centrifugation 5-ml. portions of the supernatant were withdrawn from one centimeter above the bottom of the tubes and their O.D. determined. The difference between the O.D. before and after centrifugation was proportionate to the number of sperm sedimented. This difference was always greater for suspensions of motile sperm. The difference between the decrease in O.D. of live and formalin-killed suspensions was assumed to result from the "downward" migration of sperm in the motile sample, and was considered to be the motility score of the sample (*cf.* van Duijn, 1963, for discussion of sperm migration rate). This score was determined by the equation

$$M = dL - dK,$$

where *M* is the motility score, *dL* is the change in O.D. ($\times 100$) of the living sample, presumably resulting from sedimentation plus "downwards" swimming of sperm, and *dK* is the change in O.D. ($\times 100$) of the formalin-killed sample representing sedimentation unaltered by motility. Under these conditions the value of *M* for 50 freshly diluted sperm varied from 3 to 41 with a mean value of 14, with 15 recurring most frequently.

EXPERIMENTS AND RESULTS

Motility by centrifugation

Motility can be rated quantitatively by finding measurable differences between motile and immobile samples of sperm that are otherwise equivalent. It was found that such differences resulted when live and dead sperm suspensions were centrifuged. Living, motile sperm sedimented faster than formalin-killed ones, and the difference could be measured as described above. A possible explanation of the

difference in sedimentation rates is that sperm oriented head "downwards" in the centrifugal field (*cf.* Rothschild, 1962) because their tails are more buoyant than their heads (Kihlström, 1958; Beatty, 1964), and, therefore, motile sperm swam "downwards" faster than dead ones sank.

The hypothesis that sperm were oriented by centrifugal force was tested in the centrifuge microscope. Formalin-killed sperm were seen to be oriented head "downwards." Living sperm moved very rapidly "downwards," but it was impossible to see any orientation because of the rapid movement of the sperm superimposed on the flashing field of the microscope, and so it remained uncertain whether or not living sperm were oriented in the centrifugal field.

The following experiments tend to support the assumption that living sperm sedimented faster than dead ones because they swam "downwards." Sperm from the pellet that formed when living sperm were centrifuged were highly active

TABLE I
*Motility and fertilizing capacity of various fractions of centrifuged sperm**

Experiment number	Motility**		Fertilizing capacity***		
	Pellet	Control	Pellet	Supernatant	Control
1	9	3	0.22	0.03	0.21
2	9	4	5.0	0.13	2.5
3	15	4	2.5	1.4	—

* Sperm were centrifuged 20 minutes at 200 g. The upper 5 ml. of the supernatant and the sperm in the pellet were then withdrawn by pipette. Pellet sperm were resuspended in sea water to the original concentration. These fractions were then tested for motility or fertilizing capacity. The original uncentrifuged suspension served as a control for aging.

** Motility score (M) is in Klett units.

*** Fertilizing capacity is the reciprocal of sperm concentration wherein just less than 100% fertilization was attained ($\times 10^{-6}$).

when viewed under a microscope. Sperm remaining in the supernatant seemed less active. When sperm from the pellet were resuspended in sea water and tested for motility by centrifugation, they had higher motility scores than control sperm of the same age, and were more effective at fertilizing eggs than either sperm from the supernatant or uncentrifuged control sperm (Table I). This could mean that sperm were improved by being packed into a pellet, or, as seems more likely, that the most active and effective ones were concentrated by centrifugation.

The experiments on aging reported below also tend to confirm that the difference in sedimentation rates resulted from motility. The proportion of live sperm sedimented (dL) was greatest at first when samples were visibly most active and gradually decreased until it equalled the proportion of dead sperm sedimented (dK) as the samples aged and became immotile (Fig. 1). Similarly, sperm immobilized at low pH or by narcosis with carbon dioxide (*cf.* Mohri and Yasumasu, 1963) sedimented at the same rate as formalin-killed sperm: that is to say, hardly at all (Fig. 2). It therefore seems likely that the formalin-killed sperm sedimented more

slowly than live ones because their motility was inhibited rather than because of some extraneous effect of the formalin.

Motility rating by centrifugation could be influenced by the sperm concentration (Rothschild, 1956a; Tampion and Gibbon, 1963). Table II compares the values obtained when the motility of the same sperm sample was determined at different concentrations. Sometimes, but not always, the more dilute sperm gave lower motility values. This may have resulted from more rapid aging by more dilute sperm (Rothschild, 1948b). Dilute sperm tended to lose motility very rapidly at first (Fig. 1), but as Gray (1928) observed, sea urchin semen samples vary. He reported that the respiration of some samples declined rapidly after dilution, while others showed some lag before beginning to decline. The concentration effect on

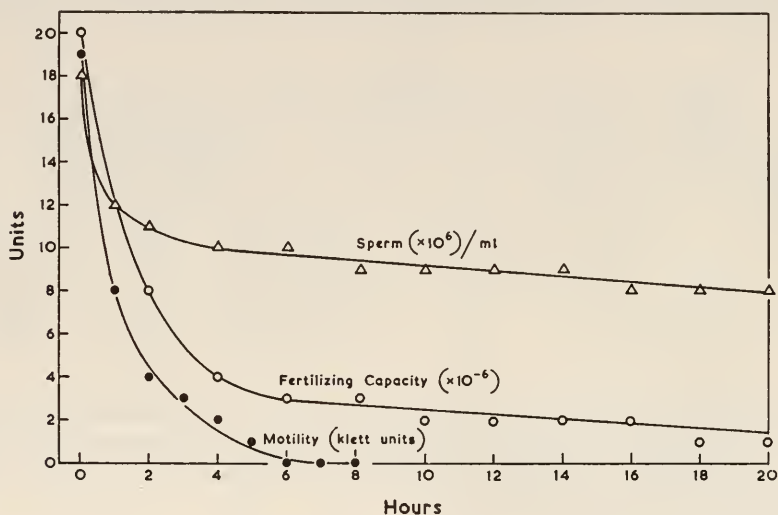


FIGURE 1. Change in sperm concentration, fertilizing capacity and motility with time. Initially *Arbacia* semen was diluted to 18×10^6 cells/ml. in 500 ml. of sea water. Periodically the suspension was stirred and portions tested for motility and fertilizing capacity. Motility is in Klett units. Fertilizing capacity is expressed as the reciprocal of the concentration wherein just less than 100% fertilization was attained (units $\times 10^{-6}$). Sperm concentration is in millions of sperm/ml. (units $\times 10^6$).

motility rating that was sometimes observed may also have reflected physical interaction between sperm (Taylor, 1952; Rikmenspoel, 1962; Tampion and Gibbon, 1963; van Duijn, 1963). The motility score used in the experiments reported below is probably valid only if sperm concentration and time after dilution are taken into account.

Sperm senescence

This somewhat quantitative method for rating motility was used to investigate the relationship between motility and the loss of fertilizing capacity by aging sea urchin spermatozoa.

In the initial experiments semen was diluted about 2000-fold in filtered sea water

and allowed to stand in 100- or 250-ml. beakers at room temperatures. Periodically the suspensions were thoroughly stirred and portions tested by centrifugation for motility, and in serial dilution for fertilizing capacity. Sperm concentration was determined by absorptiometry at each interval and confirmed occasionally by direct counts.

Under the conditions of these experiments (25 in all) sperm concentration as well as motility and fertilizing capacity declined with time (Fig. 1). In different

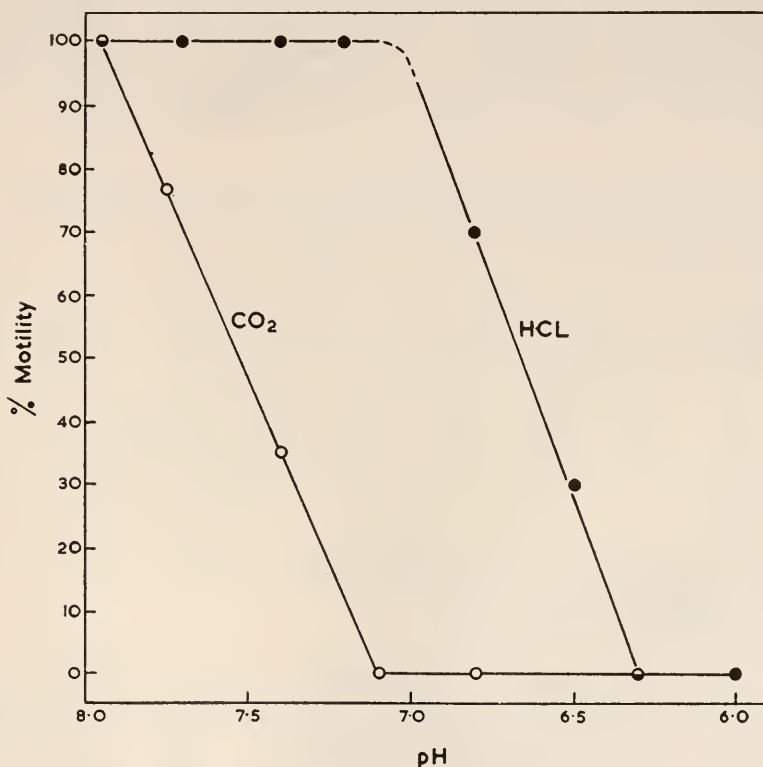


FIGURE 2. Effects of acid and carbon dioxide on *Arbacia* sperm motility. Sperm were shed into 250 ml. of sea water to 18×10^6 sperm/ml., then divided into three equal portions. One portion served as control, the second was acidified by bubbling with 6.8% CO₂ in air, and the third was acidified with HCl. The motility of the three portions was determined simultaneously by centrifugation. The process was repeated at different pH values with fresh sperm shed from the same male. The data are presented as percentage of the control sperm motility.

samples the average rate of decrease in concentration varied from 0.25×10^6 sperm/ml. lost each hour to 5×10^6 sperm/ml. lost each hour. There was no evidence that the loss of sperm resulted from sedimentation or the adherence of sperm to the walls of the container. The decrease in sperm concentration had to be considered in motility and fertilization capacity determinations, so concentration was determined and new portions killed for motility determination at each time interval.

Motility and fertilizing capacity of sperm declined most rapidly soon after dilution and more slowly later on (Fig. 1). Motility became imperceptible, as rated by the centrifuge method, after about six hours. Such "immobile" sperm were seen under the microscope to be twitching slightly but not progressing. Fertilizing capacity, on the other hand, persisted for 30 to 40 hours after dilution (as determined directly in three experiments and estimated from semilog plots of the data from the other experiments). Actively moving sperm were observed trapped in the jelly coat of eggs inseminated with dilutions of aged, apparently immotile sperm. This suggested that sperm were stimulated to renewed activity under the conditions of fertilization. The possibilities were considered that such rejuvenation could have resulted from (1) further dilution (Rothschild, 1956a) or (2) stimulation by substances exuding from eggs (Hathaway, 1963).

TABLE II
The effect of sperm concentration on motility determination by centrifugation*

Dilution		Sperm sample								
		1	2	3	4	5	6	7	8	9
1	C**	22	33.5	61	36	47	19	31	17.5	19
	M***	15	17	7	6	25	19	7	15	15
2	C	11	20.5	29	18	18	14	16.5	16	17
	M	15	6	6	6	6	15	7	9	15
3	C	—	9.5	13	9	8	11	7.5	14	16
	M	—	6	3	6	4	8	7	10	17

* Semen was serially diluted and tested for motility.

** Concentration is in millions of sperm per ml.

*** M = Motility score in Klett units.

Sea urchin sperm are known to be stimulated to a burst of activity by dilution in sea water (*cf.* Mann, 1964, p. 343). The rejuvenation of sperm suggested above could have resulted from such a "dilution effect." Sperm activity in the aged samples could have been suppressed by exhaustion of oxygen or accumulation of respiratory CO₂ with a concomitant fall in pH (Rothschild, 1956a; Mohri and Yasumasu, 1963), and then restored by further dilution. In order to test this hypothesis, air was bubbled through sperm suspensions as they aged (two experiments). Aerated sperm lost motility at a uniform rate while the control sperm lost motility more rapidly at first and more slowly later on. Some motility persisted in the control samples for several hours after the aerated ones lost perceptible motility. Concentration and fertilizing capacity declined more rapidly in aerated than in control suspensions. The pH was constant (8.0) in the aerated sample but fell to pH 7.6 in the controls during the first hour, and then remained constant. Rothschild (1956b) reported that a pH increase from 7.84 to 8.00 resulted in a 400% increase of the respiratory rate of *Echinus esculentus* sperm. Mohri and Horiuchi (1961), on the other hand, reported that Japanese sea urchin sperm (*Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus* and *Anthocidaris crassispina*) were little

affected by varying the pH from 7.0 to 8.5. In order to evaluate the significance of the observed drop in pH on *Arbacia* sperm suspensions the effects of acid and carbon dioxide on motility were compared with the centrifuge method for rating motility. In three experiments the hydrogen ion concentration of freshly shed sperm suspensions was adjusted to a given pH in the range of 8.0 to 6.0, either with HCl or by bubbling 6.8% CO₂ in air through the suspension. The motility of acidified sperm was then compared with control sperm and results expressed as the percentage of control motility remaining (Fig. 2). The results clearly indicated that CO₂ inhibited motility at hydrogen ion concentrations which had little or no effect on *Arbacia* sperm motility. The fall in pH from 8.0 to 7.6 observed in the preceding experiments on aging could have appreciably suppressed motility if it resulted from accumulating CO₂.

It was of interest to see if the fertile life of diluted sea urchin sperm could be prolonged by depressing their motility (cf. VanDemark, Koyama and Lode, 1965). It has long been known that sperm immobilized with acid or CO₂ can fertilize eggs after dilution in sea water (Cohn, 1918). In four experiments sperm were diluted and allowed to age in sea water adjusted to pH 6.5 or 7.0 with HCl or CO₂. The pH was adjusted occasionally so that it did not fluctuate more than 0.1 unit from the desired value. Periodically samples were tested for sperm concentration and fertilizing capacity. In these experiments the fertilizing capacity of acidified sperm remained at the high initial value for about 24 hours and then declined rapidly so that all sperm became infertile after about 32 hours. Control sperm (in sea water, pH 8.0–7.8) lost fertilizing capacity rapidly in the first few hours and then more slowly until all fertilizing capacity was lost at about 32 hours (cf. Fig. 1). In one experiment sperm were completely immotile in sea water adjusted to pH 6.5 with either HCl or CO₂ (cf. Fig. 2). The fertilizing capacity of both acidified samples was initially about 25% less than the control value, but persisted undiminished for 22 hours and then declined rapidly in both samples. At pH 7.0 (three experiments) sperm in sea water with HCl were slightly motile while those in sea water with CO₂ were immotile. The fertilizing capacity of both samples remained the same as the initial control value until the rapid decline began after about 24 hours. The fertilizing capacity of sperm in HCl-adjusted sea water began to decline about two hours before the ones in CO₂-adjusted sea water in all three experiments. In these experiments the sperm concentration diminished less rapidly in the acidified suspensions than in the control suspensions. In one experiment the control sperm were all gone after 14 hours, while the concentration of sperm held at pH 7.0 did not diminish in 32 hours. In a fifth experiment 50 units of penicillin per ml. were used to control bacteria (Mohri, 1957). In this six-hour experiment, cell loss was reduced by penicillin to the level observed in the immobilized suspensions, while the concentration of sea water control sperm diminished 0.26 million cells per ml. hour. The penicillin had a marked detrimental effect on motility and fertilizing capacity, however, so the experiment was discontinued.

A second hypothesis accounting for the rejuvenation of aged sperm is that substances exuding from eggs could reactivate "immobile" sperm (Hathaway, 1963), just as the surface of the chorion near the micropyle of fish eggs activated motionless sperm (Yanagimachi, 1957). It proved unfeasible to test this hypothesis, however, because the water in which eggs had stood caused irreversible agglutination

of formalin-killed sperm (*cf.* Tyler and Bishop, 1963) and thus rendered motility rating by the centrifuge method unreliable.

CONCLUSIONS

Senescence of *Arbacia* sperm in the experiments reported above occurred in several phases. Soon after dilution, sperm motility, fertilizing capacity and concentration declined rapidly. After about six hours motility became imperceptible by the rating method used here. Thereafter the concentration and fertility of the remaining sperm declined much more slowly (Fig. 1). Ultimately after 30 to 40 hours, all fertility was lost.

Fertility and sperm concentration both declined most rapidly soon after dilution when motility was greatest (Fig. 1). This initial rapid decline was prevented by suppressing motility with hydrogen ions and/or carbon dioxide. It therefore seems likely that the loss of sperm from suspension and the initial diminution of fertility were both associated with exhaustion of energy supplies. Afzelius and Mohri (1966) demonstrated that reduction in mitochondrial cristae apparently resulted from the catabolism of phospholipids and suggested that sea urchin sperm might burn up structural elements for energy. It is conceivable that the observed decrease in sperm concentration resulted from such autolysis.

Bacterial contamination could have been involved in the destruction of sperm and the loss of fertility by the remaining ones. The decline in sperm fertility and concentration, however, was most rapid soon after dilution when bacterial contamination should have been minimal, and the rate of decline diminished later when bacterial effects should have been more pronounced. It seems more likely that bacteria had a role in the final phase of sperm aging, when all fertility disappeared from the samples, rather than in the striking initial changes. This final loss of fertility was probably due to factors other than exhaustion of energy reserves, since it occurred at about the same time whether motility was suppressed or not (*cf.* Mann, 1964, p. 349).

Gray (1931) emphasized that sperm suspensions are heterogeneous populations of cells. He recognized this heterogeneity in his data as the varying rate of decline of respiration as sperm aged, and attributed it to physiological variability in the composition of individual cells. The data presented above can also be interpreted in this way.

Figure 1 suggests that the semen samples consisted of a population of short-lived sperm that disappeared during the first phase of aging and a second population that persisted through the second phase. Perhaps the short-lived sperm exhausted their energy supplies rapidly while the second population maintained a reserve of energy. Slow-speed centrifugation is apparently a useful method for separating sperm according to motility. It therefore presents a tool to investigate further physiological variability within semen samples and the significance of this variability to studies of gamete physiology and development.

I wish to thank the Will Corporation and, specifically, Mr. Willis Noland for the use of a microscope for the summer. I am indebted to Dr. Yukio Hiramoto for his help in the examination of sperm in the centrifuge microscope, and to Dr. Ralph

Hathaway for a pressure siphon used for withdrawing samples from centrifuge tubes while leaving the sediments undisturbed.

SUMMARY

1. The aging of semen was investigated in terms of sperm motility. A method for rating motility was devised. It was based on the observation that motile sperm sedimented faster than formalin-killed controls when subjected to low-speed centrifugation.

2. The aging of semen was found to involve the loss of motility, the loss of fertilizing capacity and disappearance of sperm from suspension. All three of these factors declined most rapidly at first and more slowly later on. The rapid loss of fertilizing capacity and the disappearance of sperm could be delayed by suppressing motility with hydrogen ions or carbon dioxide.

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THE REPRODUCTIVE CAPACITY OF ARTEMIA SUBJECTED TO SUCCESSIVE CONTAMINATIONS WITH RADIOPHOSPHORUS ^{1,2}

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The dangers of contaminating the environment with radioisotopes or other deleterious substances have received much publicity. Often the main biological consideration has been the number of adult individuals of susceptible species seen or caught during a particular season. These numbers are then compared with records of other years in an attempt to infer damage or to claim unimpairment. Unfortunately, adult abundance equalling that of an area before exposure does not necessarily indicate recovery from genetic damage to the population. The reproductive capacity of an organism may be adequate to compensate for infecundity and the death of immature stages. Therefore, to reveal the consequences of contamination, the quantitative aspects of natality and survival to maturity must be studied.

In our laboratory, *Artemia* populations are being used in studies of induced changes in the components of biological fitness after subjection to a variety of agents. Results for the last four years of an 8-year study of radiophosphorus effects are reported below. Most notable is the consistent demonstration that the number of adults can be identical in different mass cultures, but the reproductive potential of populations with different ancestral histories differs considerably.

The fitness components obtained from *Artemia* pair mating tests over a four-year period following radioisotope exposure of ancestors were reported earlier (Grosch, 1962). Subsequently several of the original populations have been subjected to further experimentation and analyses which reveal persistent inferiority of reproductive performance in pair matings. In addition we have determined whether subpopulations can survive repeated contaminations, an important problem in radioecology. Our results indicate that species density or standing crop data can be misleading. Although adults in an experimental culture may be abundant, their reproductive potential may be inadequate to withstand further damage by radiations.

MATERIALS AND METHODS

Two isotopes, Zn⁶⁵ and P³², differing in rays, half-life, and metabolic fate, were selected for *Artemia* experiments because of their persistence in Columbia River food chains (Foster and Davis, 1955). All Zn⁶⁵ populations are now extinct, not

¹ These experiments were begun with the help of summer assistants supported at the Marine Biological Laboratory by U. S. Atomic Energy Commission funds. Currently the author receives support from U. S. Public Health Service research grant ES-00044, Division of Environmental Engineering and Food Protection.

² Contribution from the Genetics Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper No. 2137 of the Journal Series.

only the 30 $\mu\text{c.}/3\text{L}$ culture of the 1962 (Grosch) report, but also several 20 $\mu\text{c.}/3\text{L}$ cultures followed subsequently. On the other hand, many of the P^{32} cultures survived. One of these is a 90 $\mu\text{c.}/3\text{L}$ culture which is providing useful data. In nature, although it comprises less than 1% of the radioactivity in Hanford wastes which contain more than a dozen different nuclides, P^{32} accounts for 40% to 95% of the radioactivity of most Columbia River invertebrates and fish. This reflects the biological demand for an element incorporated into genetically important nucleic acids and energy storage-transfer systems.

The three-liter mass cultures derived from the diploid amphigonic strain of *Artemia salina* have been maintained in cylindrical gallon jars for nearly a decade at room conditions in Woods Hole. During the summer they have received 1 ml. of yeast suspension daily. During the winter they typically evaporated to less than one-third of the summer volume. In spring the cultures were reconstituted by adding distilled water to dissolve salt encrustations and activate cysts. Putrefying dead algal masses were removed as soon as *Artemia* emergence seemed complete. With such attention a population of 250 to 300 adults has quickly developed in every three-liter container except in strains nearing extinction. The control cultures maintained simultaneously with experimental cultures under identical conditions, were derived from ancestors which have never been exposed to radioisotopes or other deleterious agents of technological origin.

Artemia culture techniques have been improved during the years in which jar populations have been maintained and studied. Although *Artemia* is an organism assumed to be exceptionally tolerant because the adults survive for days in a wide range of salinities, previous work (Grosch, 1962) indicated that reproductive performance was improved by increasing the salinity above that of sea water. From their own experience other geneticists (Goldschmidt, 1952; Bowen, 1962) decided to culture *Artemia* in water saltier than sea water. Prior to 1962 an increase in salinity for our mass cultures resulted only from the slow process of evaporation. Since 1962 NaCl has been added routinely to mass cultures. Present practice is to bring them to 50 grams of added NaCl per liter before maturation of the summer's first generation. In 1964 and 1965 water of increased salinity was used also for pair mating tests. Bowen's routine medium was adopted, 50 grams of NaCl per liter of filtered sea water.

Each subpopulation to be subjected to an addition of radioisotope was obtained by transferring 20 adult pairs to a gallon jar containing three liters of brine. For pair mating studies, 15 young pairs were transferred from mass culture as soon as the male had clasped the female. Since arbitrary matching was not practiced, the pairs studied are representative of those contributing to the future of the population from which they were withdrawn. Each pair was placed in its own quart jar. The average number of days between the transfer to quart jars and the death of members of mated pairs is taken as a measure of adult life span.

Jars containing parental pairs and the jars to which their broods were transferred were maintained under constant illumination from a bank of fluorescent tubes. The water temperature ranged between 25° and 28° C. All jars were examined daily at the time of feeding with yeast suspension (0.3 ml.). When present, cysts were filtered, dried and resuspended in filtered sea water for emergence tests.

RESULTS AND INTERPRETATIONS

A persistent population descended from ancestors exposed to 30 $\mu\text{c.}$ of P^{32} produced hundreds of adults, generation after generation and year after year in mass culture. However, subcultures were unable to survive a second 30- $\mu\text{c.}$ dose until four years or a minimum of 12 generations had elapsed. Furthermore, as shown in Figure 1, additional years and generations passed before subcultures managed to survive a third 30- $\mu\text{c.}$ dose of P^{32} . Evidently the carrying capacity (300 adults) of a three-liter culture was easily achieved by *Artemia* of experimental lineage. Differences between strains were revealed only by investigating reproductive performance. For this purpose we employ isolated parental pairs.

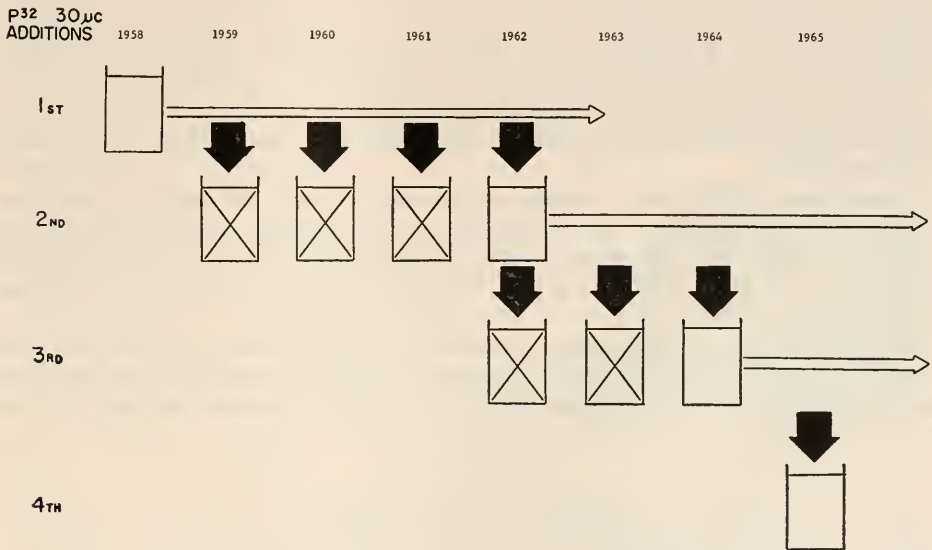


FIGURE 1. The origin and subsequent fate of three-liter experimental cultures of *Artemia* which received 30- $\mu\text{c.}$ doses of P^{32} . The black arrows indicate subcultures given radiophosphorus. Cultures marked with an X did not survive. After three months no adult offspring have appeared in the 1965 "fourth addition" jar and no horizontal arrow representing continued survival of the strain is shown. The subculture of control origin which received the first addition of P^{32} was discarded in 1963.

An adult female can exhibit both oviparity and viviparity (Lochhead, 1961). In pair mating tests, control females were not strongly inclined toward oviparity. They gave birth to relatively more live young and deposited fewer cysts than did females from other strains tested. Table I summarizes records for the last four years in which the percentage of zygotes encysted has been lowest for control parents year after year. In addition, for the years 1962 and 1965, the lowest percentage of emergence or "hatchability" of the cysts was found in the controls. An interesting feature of the experimental results has been the steady climb in percentage of emergence. Originally in 1959, one year after the population had experienced its first radiophosphorus exposure, emergence was less than 25%. In

TABLE I
Cyst deposition and emergence of larvae from four years' records of pair matings

Strain tested	% of zygotes encysted	1962 % emerged	% of zygotes encysted	1963 % emerged	% of zygotes encysted	1964 % emerged	% of zygotes encysted	1965 % emerged
Control	44.19	30.66	51.35	49.26	30.47	65.93	26.40	27.60
30- μ c. Experiments First P ³² addition	65.30	36.17						
Second P ³² addition			76.23	40.32	62.32	42.82	96.04	66.94
Third P ³² addition							87.64	63.32
90- μ c. P ³² Single dose	None		None		58.49	26.82	48.19	58.09

1965 emergence has reached a high of 66.94% for a strain which has received two doses of P³².

The results reflect the performance of all members of the sample rather than that of only a few females. Every female which produced young also yielded cysts, typically as her first brood and often as her last. This contrasts with the records in our 1962 paper in which only controls showed a majority of the females depositing cysts.

Control pairs tended to produce a greater number of broods. In addition there were more zygotes per brood and of these more survived to adulthood than survived in tests of progeny from exposed ancestors. Table II summarizes the life span

TABLE II
The life span and fecundity of parents from pair mating tests along with survival and sex ratio of offspring

	Adult life span in days		Broods per ♀	Zygotes voided		% surviving to adult	Mature adults per ♀	Sex ratio ♂/♀
	♀	♂		per brood	per ♀			
1963								
Control	16.3	14.2	1.2	272.78	327.34	29.91	97.91	0.97
30- μ c. second addition	16.6	21.9	1.4	130.70	182.98	12.85	23.51	0.95
90- μ c. single dose	12.7	12.8	0.3	74.33	22.30	0.58	0.13	1.33
1964								
Control	60.45	66.65	12.25	187.35	2295.04	73.49	1686.62	0.80
30- μ c. second addition	33.62	44.38	2.87	133.98	384.52	49.93	181.99	0.88
90- μ c. single dose	30.60	31.60	5.7	177.84	1013.68	17.07	173.35	0.99
1965								
Control	55.45	52.18	10.18	157.17	1599.99	59.45	951.18	0.85
30- μ c. second addition	21.92	31.17	3.83	106.26	406.96	47.70	194.12	0.91
30- μ c. third addition	30.71	36.71	4.36	109.59	477.81	56.71	270.97	0.81
90- μ c. single dose	32.08	38.00	3.63	81.45	295.66	68.71	203.15	0.90

of parents and their reproductive performance for 1963 through 1965. Included are the sex ratios for adult progeny. Females are favored, except in 1963 for the 90- μ c. culture which was then not doing well. These results differ from the earlier sex ratios which favored males in many irradiated strains (Grosch, 1962).

Sex ratio can be discounted but the problem is to decide if improvement in any other aspect such as adaptive value is involved in surviving an additional radio-isotope contamination. Adaptive value can be defined as the relative capacity of carriers of a given genotype to transmit their genes to the gene pool of the following generation. It may be calculated by dividing the mature adults per pair of treated ancestry by the number of mature adults per control pair. The adaptive values for the 90- μ c. strain have shown regular improvement from nearly zero in 1962 through 0.001, 0.10, to 0.21 in the last three years. On the other hand adaptive values have varied for the 30- μ c. doses, due partly to variability in control performance which is taken as unity in deriving the value. On this basis an average of 191.99 live adults in 1964 does not compare so well with 194.1 in 1965 when respective control performance is used as the basis for calculating A.V.'s of 0.11 and 0.20. Possibly prolonged patterns of weather are reflected in our data; 1964 and 1965 were different types of summers.

The reproductive advantage of controls, expressed as mature adults produced per parental pair, depends in part upon the length of time adults survive in quart jars. In 1963 before the Bowen medium was adopted for pair mating tests, the ratio of control to "second addition" progeny, 97.9:23.5, was about 4:1. In 1964 the ratio was about 9:1 and in 1965 the ratio was about 5:1. Part of the difference comes from an increase in survival (mean adult life span) from less than three weeks to approximately two months. Although the poorest experimental groups are now attaining life spans equivalent to or exceeding control values of previous years, the difference which was not apparent in 1963 has become pronounced. Controls are now living nearly twice as long as adults from experimental populations.

With increased life span, improvement in the number of offspring per female was inevitable because broods have been deposited with regular frequency. The raw data are too extensive for tabulation here. Analysis of 1965 brood frequency showed no significant difference in the averages of the interval for control (3.00 ± 0.91), second (3.20 ± 0.27), and third addition (3.80 ± 0.94) tests. This pattern in which females void broods every third or fourth day has been evident in our records since 1959. In 1963 when pair matings were still maintained in ordinary sea water the control interval was 3.86 ± 0.06 days and for second addition pairs 3.25 ± 0.30 days. A striking and unexplained deviation in the pattern has appeared for the 90- μ c. strain. In 1964 the interval averaged 3.07 ± 0.02 days. In 1965 the strain showed a longer period between broods; the interval of 6.80 ± 0.98 days differs significantly from all others cited. These frequencies which concern only the pattern between the first and last brood voided cannot be inferred directly from the number of broods per female shown on Table II. The time elapsing between isolation of pairs and deposit of first brood varies as does the period intervening between last brood and death.

Another influence of life span appears to be upon brood size but the relationship is not simple. In young adults brood size is correlated with the increasing size of

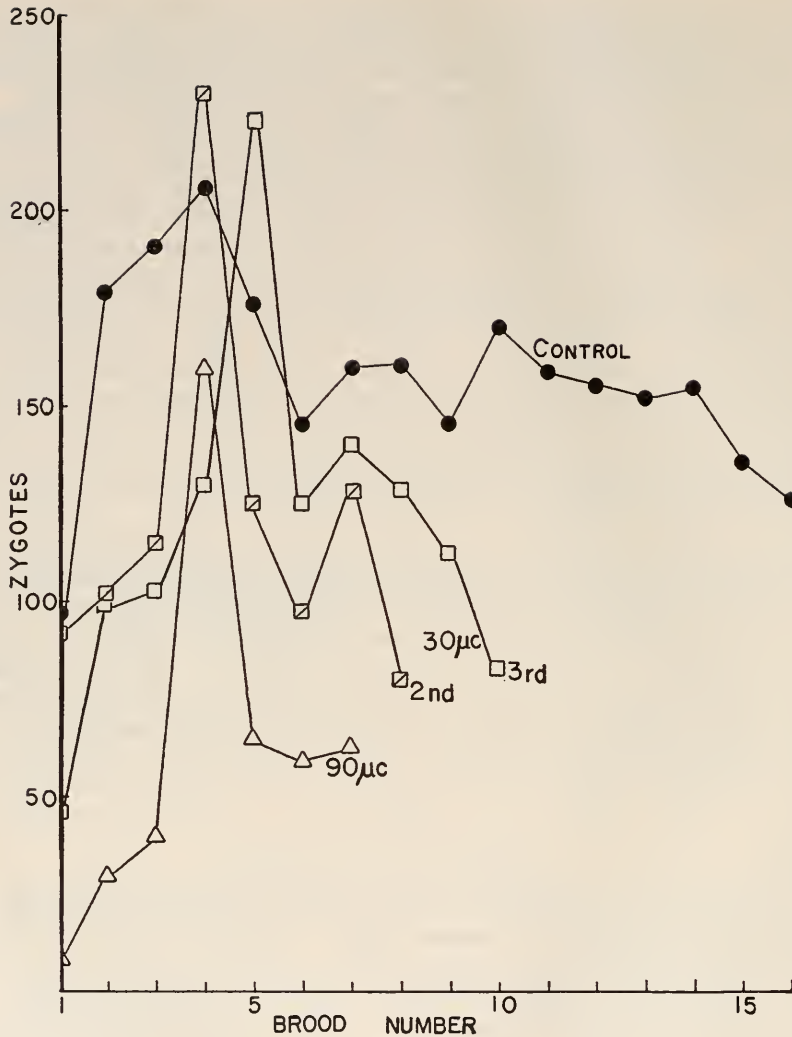


FIGURE 2. Brood size plotted against the sequence of brood deposit. Each curve represents the reproductive record of the individual female surviving for the longest period of time in its respective group of 1965 pair mating tests. The solid circles indicate the control example. Triangles represent values for the long-lived individual from a culture which received a single 90- μ c. dose of P^{32} in 1959. Squares mark values for the examples from 30- μ c. addition experiments. The number of successive doses in the history of the culture is indicated by a designation at the end of the curve, 2nd = 2 and 3rd = 3 doses.

the female. Subsequently brood size declines, presumably as a reflection of aging or because of debility associated with impending death. The clearest demonstration of this pattern was obtained by plotting brood size against brood sequence for each individual female. A peak and subsequent decline is characteristic of records for all females surviving 15 days, the time usually required for voiding about five

broods. Plotting the average brood size is an unsatisfactory procedure in this case. Particularly for experimental females the pattern of fecundity tends to be obscured in pooled data by the broods contributed by short-lived females.

In order to reveal potentialities in fecundity, patterns of brood size for females of maximum longevity from each of the populations sampled by pair mating tests in 1965 are shown in Figure 2. Peak productivity was achieved by the fifth or sixth brood. The subsequent decline was pronounced for females of experimental ancestry but moderated by a long plateau for the control sample. As evidence that the example is representative of the control pattern we can state that 7 of the first 10 points are nearly identical with the respective average values, and the remaining three points lie within one standard error of the pertinent control values. The control sample which averaged 10 broods before death (Table II) contained many individuals which survived beyond the period of maximum fecundity. On the other hand, experimental females which averaged only 3 or 4 broods before death probably failed to reach their maximum fecundity.

Although deficient, the fecundity of "second addition" pairs has not varied in a manner which would explain the response of the mass culture to additional isotope exposures. A possible key may be the survival to adulthood. These percentage values fit into a pattern:

Year	1959	1960	1961	1962	1963	1964	1965	Addition
Survival	24.4	27.6	57.6	61.0				First
					12.8	49.9	47.7	Second

Not until 1962 was a second addition tolerated. Survival to adulthood was reduced severely by the treatment and a third addition was not tolerated until the moderate recovery demonstrated in 1964 had occurred.

DISCUSSION

A twice-repeated series of P³² doses had indicated that a single addition of 90 μ c. approached the threshold dose for population extinction (Grosch, 1962). From the simple standpoint of dose arithmetic it seemed possible that subcultures from a strain that had survived 30 μ c. should be able to withstand additional doses. This was not the case, and pair mating studies revealed severe genetic damage to reproductive ability from one 30- μ c. treatment. Additional equivalent doses were tolerated only after a period during which the germ plasm presumably underwent a partial purge.

The pair mating tests summarized above reveal that maintenance of populations numbering 300 adults amounts to only a small fraction of the control potential. For example, if 300 adults comprise 150 pairs each capable of providing 951 mature offspring (1965 average), the potential number of offspring amounts to the product, 142,650. However, when the carrying capacity determined by a density-dependent process is 300, only a fraction of the reproductive potential can be utilized. This fraction $300/142,650 = 0.002$ or 0.2%. By the same reasoning, only 1% of the "second addition" culture's potential is required to maintain the ceiling level of 300 in a three-liter culture. In the past, a number of cases was observed in which 10% to 30% of the reproductive potential was used to maintain 300 adults in a

three-liter culture. This type of situation proved precarious and many such cultures became extinct. Conceivably even more extreme situations may occur in nature, and in unfavorable circumstances 80% to 100% of the reproductive potential of an organism may be required to maintain the frequencies of adults found in census.

As pointed out by Grant (1963), we don't really have enough quantitative information at present about the actual number of genetic deaths a population can tolerate and still survive under various conditions. Possibly more than 50% of the *Artemia* reproductive potential must be held in reserve to buffer populations against extinction. If so, in natural situations impaired by human activities, other organisms may be balanced even more precariously than *Artemia*. Actually *Artemia* may have an advantage over most animals. Possibly when shrimp populations reach the ceiling level their excess productivity can be switched into encysted zygotes rather than expended in juvenile mortality.

Fertility requirements are very high, usually too high, for a species acquiring many deleterious two-allele loci with high selective differentials. Several geneticists (Lerner, 1958; Wallace and Dobzhansky, 1959; Grant, 1963; Wallace, 1963) have speculated about the number of offspring which must survive if a population is to avoid extinction following the induction of simple dominant or recessive lethals. The maximum number of offspring is limited by the number of functional eggs produced per female. In this attribute *Artemia* exceeds domestic animals and most insects. On the other hand, *Artemia* fecundity is not particularly exceptional when compared with the range for fish and aquatic invertebrates (Altman and Dittmer, 1962). The cost in segregation of inferior homozygotes may be met by a fecund organism, or the price may be reduced by series of multiple alleles or by numbers of independently assorting interchangeable genes (Grant, 1963).

An alternate approach is to view lethality as the product of lethal gene combinations rather than the product of lethal genes (Mayr, 1963). The genes which interact harmoniously in the population's gene pool were brought together by natural selection acting over a long period of time. Disharmonious combinations can follow the induction of genic diversity. Recently bichromosomal synthetic semilethals have been demonstrated in *Drosophila pseudoobscura* (Dobzhansky *et al.*, 1965). Individuals homozygous for specific second and third chromosomes showed viability down in the semilethal range. Conceivably disharmonious interactions also can occur in heterozygous genotypes. So many beneficial effects of heterozygosity have been described that we are too inclined to regard all heterozygosity as good, but for example, a loss of epistatic balance among interacting loci can override the beneficial effect of high heterozygosity (Mayr, 1963).

To date we have been unable to demonstrate increased genetic fitness in irradiated *Artemia* such as shown by Wallace (1956) in *Drosophila* and by Crenshaw (1965) in *Tribolium*, but admittedly a demonstration of the phenomenon may require a more inbred strain of shrimp than is yet available. Also our testing has been limited to a particular season. Possibly in certain seasons (or years) it might be possible for experimental *Artemia* to equal or exceed the controls in fitness. On the other hand, Sokal and Huber (1963) reported heterozygote intolerance to crowding in one *Tribolium* experiment, and Sankaranarayanan (1965) found *Drosophila* subpopulations plateauing at 70 to 75% viability in x-ray experiments. This level achieved in five generations after cessation of irradiation

indicated more rapid recovery than we have observed with *Artemia*, but gives no evidence of superiority of irradiated strains.

In prolific mass cultures, crowding is more pronounced than that experienced by broods in quart jars. Until this year survival to adulthood in experimental broods in quart tests has differed obviously from control values, and any effect of crowding has been obscured. However, even the 90- μ c. strain has improved to the degree that its fewer zygotes per brood (81.45) enable compensation in a higher percentage of survival to adulthood in the 1965 results of Table II. When 68.71% survival was compared with the control's 59.45%, the contingency chi square calculated from the raw data was 11.48 with $P < 0.001$. On the other hand, 106 to 109 shrimp per quart jar do not provide a situation significantly different from the control's 157 per quart. In these cases survival to adulthood does not exceed that of controls.

Fly crowding experiments furnish some interesting parallels and differences. Survivorship near 60% can be demonstrated through a wide range of fly densities for a variety of strains, and in house fly experiments Sullivan and Sokal (1963) quote 67% survivorship for a density of 160 per bottle, considered "normal conditions." Competition became impressive only at 1280 flies per bottle and extreme at 2560 per bottle. This was reflected by "negligible" adult emergence, 1% or less in some strains (Bhalla and Sokal, 1964). Thus normal density in fly cultures gives survival similar to that in *Artemia* brood tests, and high density fly experiments more nearly resemble what may happen in prolific mass cultures of shrimp. Moderate crowding to give selection pressure of intermediate intensity of *Tribolium* provides another example of insect survivorship within a 58 to 78% range (Sokal and Huber, 1963).

Differences from insect results derive from differences in growth pattern and become pronounced as development nears maturity. Size is determinate for holometabolous insects and indeterminate for shrimp. In crowded dipteran populations the usual response is a maintenance of numbers accompanied by a reduction in size of individuals (Sullivan and Sokal, 1963). Brine shrimp respond by repressed rate of growth and delayed maturity of part of the group or brood (Grosch, 1962), suggesting a feedback phenomenon such as reported by Rose (1960) for fish and Amphibia. Unfortunately, for purposes of comparison, cannibalism occurs among crowded fish.

If adults function as growth suppressors, their longevity could interfere with a turnover of generations. No data are available on the effects of crowding on the longevity of *Artemia* adults but with moderate fecundity and good potential survival to adulthood, parents need live only long enough to produce a brood or two in order to maintain a mass population in three liters. Our life span data concern isolated pairs. Although present techniques prolong adult survival, individual examples of extreme longevity, such as reported by Lochhead (1941) and Bowen (1962), have not been obtained. However, frequent transfer is necessary in order to assess the reproductive performance of pairs and under such circumstances even inanimate objects like cafeteria tumblers have a predictably limited life span (Brown and Flood, 1947).

SUMMARY

1. This paper is a progress report on four additional years of studying *Artemia* in and from mass cultures to which radioisotope has been added.

2. Although the number of adults seen in mass cultures may be equivalent, subcultures of control and experimental strains react differently to radioisotope additions. Strains descended from ancestors exposed to P^{32} do not necessarily survive a second dose even though total dosage does not exceed the extinction dose given as a single addition. A period of recovery involving a passing of generations must intervene. Depending upon culture conditions, this may involve two to four years.

3. Pair mating tests revealed that in comparison with controls, experimental strains:

- A. Have a shorter life span.
- B. Deposit fewer zygotes per brood.
- C. Deposit more of their developed zygotes as viable cysts.
- D. Show poor survival to adulthood except when crowding is mitigated by low fecundity.

4. Because the frequency of brood deposit has been regular in both control and experimental strains, an increase in total progeny has accompanied increased life span. The one exception to an interval of three to four days between deposits occurred in 1965 records of a strain recovering from a near lethal dose.

5. The sex ratios among adult progeny from pair matings now favor females in all strains.

6. On the basis of pair mating tests, maintenance of mass cultures at an observed level of 300 adults per three liters requires only 0.2% of the reproductive potential of controls. Cultures of experimental origin utilize 1% or more of their potential to maintain the same total.

7. The proportional number of larvae surviving to adults may be critical in determining whether or not a strain can tolerate another exposure to a radioisotope.

8. Although general comparisons may be drawn to crowding experiments in insect populations, a complete parallel is impossible because of the shrimp's indeterminate growth pattern (although *Artemia* populations are inversely density-dependent).

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SOME FUNCTIONS OF THE URINARY BLADDER IN A CRAB

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The antennary glands of crabs generally are ineffective as organs of osmoregulation inasmuch as the urine they produce remains isosmotic with the blood under conditions of hypo- or hyperosmotic stress. On the other hand, as indicated in the reviews by Lockwood (1962) and Potts and Parry (1964) the probable primary function of these renal organs is ionic regulation. Of particular interest is the high concentration of Mg^{++} attained in the urine of crabs during immersion in hypersaline water.

Prosser *et al.* (1955) demonstrated in the shore crab, *Pachygrapsus crassipes*, dramatic increases in urine Mg^{++} but decreases in urine Na^+ as the environmental salinity increased. This, they attributed to competition between Na^+ and Mg^{++} for transport across the membranes of the antennary gland, Mg^{++} prevailing. Green *et al.* (1959) observing a similar phenomenon in two species of *Uca* also suggested that Mg^{++} and Na^+ compete for transport and that active movement of Na^+ is reduced by such competition under the high Mg^{++} load found in hypersaline water. These authors as well as Riegel and Lockwood (1961), who observed the phenomenon in *Carcinus*, considered but rejected direct Mg^{++} - Na^+ exchange as the mechanism of concentrating Mg^{++} at the expense of Na^+ . Whatever the mechanism, the phenomenon seems to be common in crabs (Gross, 1964; Gross *et al.*, 1966).

Gross (1964), examining a series of crabs from aquatic, amphibious and terrestrial modes of life, revealed that animals showing high degrees of terrestriality tended to concentrate Mg^{++} more highly in the urine than the more aquatic crabs. An exception was the terrestrial *Gecarcinus lateralis* which is the only brachyuran crab examined to date incapable of concentrating urine Mg^{++} at the expense of Na^+ . Still, it was shown that high urine Mg^{++} does not necessarily reflect strong Mg^{++} regulation in the blood. For example, the urine Mg^{++} of the amphibious *Uca* was more than three-fold that of the aquatic *Cancer*, yet the blood Mg^{++} concentrations of these two species were about the same.

Gross and Marshall (1960) demonstrated that the concentration of Mg^{++} in the urine of *Pachygrapsus* is independent of the Mg^{++} influx and in some way a function of the osmotic concentration of the external medium. This phenomenon also was demonstrated in *Cardisoma carnifex*, *Varuna litterata* and *Sesarma meinerti* (Gross *et al.*, 1966).

The above described phenomena lead to the following questions: (1) What is the relationship between Mg^{++} concentration in the urine of a crab and the amount of Mg^{++} it excretes? (2) By what means does a crab immersed in a Mg^{++} -free medium of high salinity concentrate Mg^{++} in its urine? (3) By what means does the urine Na^+ concentration become reduced as the urine Mg^{++} concentration elevates when the animal is transferred from dilute to concentrated sea water?

The present investigation produces evidence that Mg^{++} concentration in the urine depends on the relative length of time the latter is held in the bladder. Mg^{++} is transported across the walls of the bladder into the urine at different rates depending on the blood Mg^{++} concentration and a direct exchange with Na^+ can take place which effects movement of water between blood and urine.

MATERIALS AND METHODS

The shore crab, *Pachygrapsus crassipes* Randall, which is a known hypo- and hyperosmotic regulator (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957) was collected at Laguna, California, and maintained in the laboratory at 15° C. in 100% artificial sea water made from the Utility Chemical Company Seven-Seas Marine Mix. Only intermolt crabs larger than 15 grams were used in the experiments. A salinity of 34.3‰ was considered to be 100% sea water. This contained the following cation concentrations: Na^+ , 455 mM/l.; K^+ , 11.5 mM/l.; Ca^{++} , 14.2 mM/l. and Mg^{++} , 55.5 mM/l. Different concentrations of sea water were attained by varying the amounts of water added to these salts. $MgCl_2$ was added to test media where the Mg^{++} concentration was to be higher than normal. Also, artificial sea water for experiments concerned with Mg^{++} depletion was made up using the proportions of Na^+ , K^+ , Ca^{++} , Mg^{++} , Cl^- and SO_4^{--} given in the tables of Barnes (1954) with the pH adjusted to 8.0. Na^+ was substituted for Mg^{++} when the latter was deleted.

Perfusion fluid used to simulate plasma and/or primary urine contained the following concentrations of ions: Na^+ , 483 mM/l.; K^+ , 7 mM/l.; Mg^{++} , 10 mM/l.; Ca^{++} , 15 mM/l., Cl^- , 520 mM/l. and SO_4^{--} 10 mM/l. This approximates the blood cation and osmotic concentration of *Pachygrapsus* when immersed in normal sea water (Gross, 1959; 1964). The Cl^- concentration approximates the mean blood concentration (517 mM/l., S.D., 11.3) of 6 crabs taken from 100% sea water. Concentrations of SO_4^{--} were estimated by difference assuming Cl^- and SO_4^{--} as the only anions and considering electro-chemical balance. Hereinafter isosmotic perfusion fluid will mean a solution made up of the above proportions but adjusted by water content to be approximately isosmotic with the blood for crabs immersed in a particular salinity. Blood osmotic concentrations for crabs immersed in different salinities are given by Gross (1957; 1964).

Immersion experiments were conducted using approximately 400 ml. of medium which was sufficient to assure complete immersion.

Osmotic concentrations of media and body fluids were determined by means of a Mechrolab, vapor pressure osmometer. Na^+ and K^+ were determined by flame photometry; Ca^{++} and Mg^{++} by ethylene diamine tetra acetic acid (EDTA) titration as previously described (Gross, 1959; Gross *et al.*, 1966); Cl^- by the method of Schales and Schales (1941); inulin was determined by the resorcinol method of Schreiner (1950).

In the range of normal sea water osmotic concentrations could be measured within a 1% error, Na^+ , about 2%, K^+ , less than 10%, Ca^{++} and Mg^{++} less than 6% and microsamples of Cl^- to less than 10%. Inulin could be measured with less than a 7% range of error.

Blood was extracted by puncturing the arthrodiol membranes at the bases of the walking legs with a glass pipette. Urine was removed from the nephropore by

means of a fine glass cannula. Since urine is clear and blood turbid, any contamination of urine with blood could easily be detected. Doubtful samples were discarded.

RESULTS

It has been shown that when *Pachygrapsus* is immersed in 100% and 150% sea water the urine Mg^{++} concentrations averaged 118 mM/l. and 204 mM/l., respectively, the corresponding urine concentration/blood concentration (U/B) values for Mg^{++} being 13.6 and 15.4 (Gross, 1959). The following experiment therefore was performed to show the role of water withdrawal in achieving the above urine Mg^{++} concentrations and U/B values. The bladders of crabs which had been immersed in 100% or 158% sea water were drained, and the animals were injected with about 0.1 ml. of an isosmotic perfusion of fluid containing approximately 6% inulin. The crabs then were reimmersed in the media from which they were taken, and after 6 or 48 hours the urine and blood were sampled for inulin analysis. Another group taken from 100% sea water was also thus treated but was kept out of water rather than reimmersed. Thus, it can be seen that the U/B values (Table I) were so low that water withdrawal cannot be a major factor in effecting

TABLE I
Inulin U/B values of Pachygrapsus

	6 hours exposure			48 hours exposure		
	No.	Mean	S.D.	No.	Mean	S.D.
100% sea water	13	1.11	0.13	10	1.92	1.29
158% sea water	10	1.16	0.24	13	1.52	0.65
Air	7	1.09	0.17	5	1.44	0.12

high Mg^{++} concentrations or U/B values. Of the mean U/B values presented in Table I only those for crabs immersed in 158% sea water or kept in air for 48 hours are significantly different from unity ($P < 0.02$). The means for the three 6-hour experiments are not significantly different. The means for the three 48-hour experiments are not significantly different, and there is no significant difference between 6- and 48-hour treatments for either salinity. The difference in urine Mg^{++} concentration in animals immersed in normal sea water compared to those immersed in hypersaline water therefore cannot be achieved by differences in water withdrawal from the urine. Further reference will be made to data in Table I later.

After 24 hours immersion in 158% Mg^{++} -free sea water, the urine Mg^{++} of 15 crabs averaged 235 mM/l. (S.D., 117) whereas the mean urine Mg^{++} of 18 crabs immersed for 24 hours in 50% sea water containing 65 mM/l. of Mg^{++} was only 20.5 mM/l. (S.D., 8.90). These data, which confirm the observations of Gross and Marshall (1960), clearly show that the ability of *Pachygrapsus* to concentrate Mg^{++} in the urine is neither a function of Mg^{++} influx nor the concentration of Mg^{++} in the medium.

Figure 1 illustrates the frequency distribution for the urine Mg^{++} concentrations of 51 crabs sampled in the field where only normal sea water was available. Figure 2 shows urine $Mg^{++} + Ca^{++}$ concentrations of crabs totally immersed in a running sea water aquarium containing 100% sea water. Small quantities of urine ($\sim 20 \mu l.$) were periodically sampled from the same nephropore of individual crabs, 10 $\mu l.$ of which were analyzed for $Mg^{++} + Ca^{++}$. Ca^{++} was not determined because of the small sample size. There it can be seen that the concentration of $Mg^{++} + Ca^{++}$ varies tremendously with time, and since urine Ca^{++} is relatively constant in concentration (approximately 20 mM/l.) with little variance (Gross, 1959), the large fluctuations in Figure 2 can be attributed to Mg^{++} . This might suggest that the

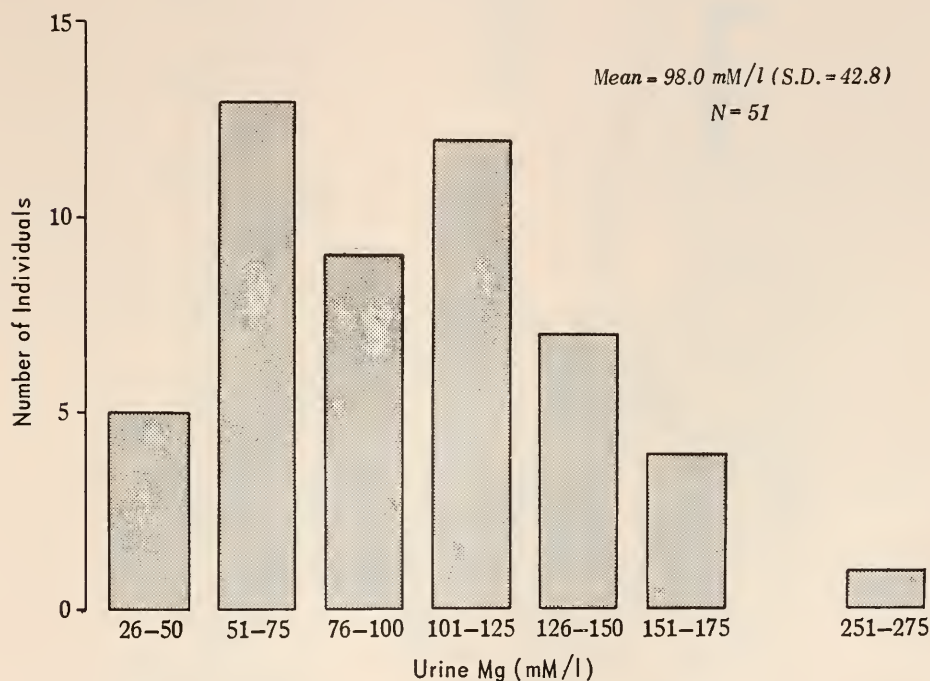


FIGURE 1. Frequency distribution for urine Mg^{++} concentrations of crabs sampled in the field where only 100% sea water was available.

Mg^{++} -transporting mechanism fluctuates in its rate of activity. However, another possibility is that the urinary bladder itself has a transporting function with respect to Mg^{++} . That is, urine entering the bladder from the labyrinth is relatively low in Mg^{++} . If the urine were held in the bladder for a prolonged period, sufficient time would permit elevation of the Mg^{++} concentration. Following bladder evacuation, then, the urine Mg^{++} should be low. When a hypo-regulating crab is immersed in hypersaline media, the water influx would be slow, the bladder would be evacuated with low frequency and urine would be held in the bladder sufficiently long to permit accumulation of Mg^{++} . On the other hand, in low salinities, water influx would be rapid in a hyper-regulating crab, evacuation of the bladder would be

frequent and no time permitted for Mg^{++} accumulation. Such a model would explain the high concentration of urine Mg^{++} for crabs immersed in 158% Mg^{++} -free sea water and low Mg^{++} concentration in urine of crabs immersed in 50% sea water containing high Mg^{++} . This would also explain the fluctuations in urine Mg^{++} shown in Figure 2. That is, low Mg^{++} concentrations would follow bladder evacuation and high Mg^{++} concentrations would precede evacuation.

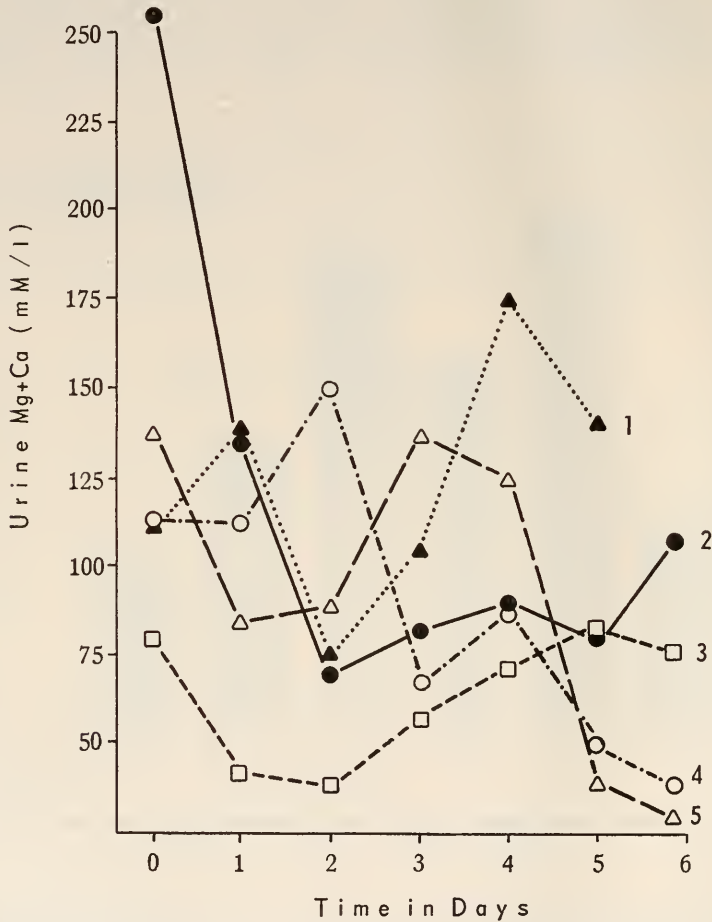


FIGURE 2. Fluctuations in the concentration of urine $Mg^{++} + Ca^{++}$ of individual crabs immersed in 100% sea water. Each symbol connected by line represents history of individual crab.

In order to test this model, one of the paired bladders of a crab immersed in 100% sea water was evacuated; the crab was then reimmersed in 100% sea water and after a given period, urine from the same bladder was sampled for Mg^{++} analysis. Thus, for 17 crabs reimmersed 2–24 hours, the mean Mg^{++} concentration was 69 mM/l. (S.D., 55) and for 16 crabs reimmersed 48–96 hours, the mean Mg^{++} concentration was 165 mM/l. (S.D., 99), the two means being significantly

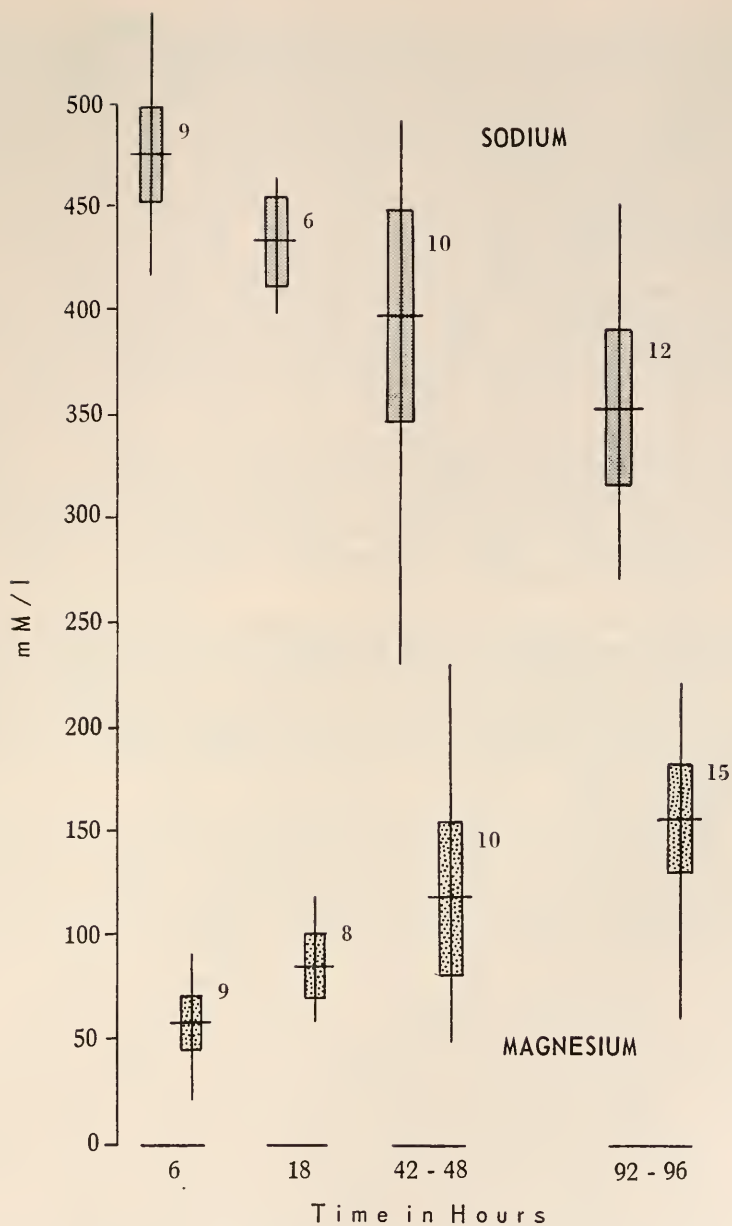


FIGURE 3. Decreases in urine Na^+ concentration accompanying increases in urine Mg^{++} concentration in crabs immersed in 100% sea water as a function of time after bladder evacuation. Mean is represented by horizontal line; range by vertical line and twice the standard error on either side of the mean by the rectangle. Numerals indicate number of cases.

different ($P < 0.01$). If it is assumed (in the above experiment) that crabs immersed for the longer periods also retain (on the average) urine in the bladder for longer periods, these data suggest that urine first entering the bladder is relatively low in Mg^{++} concentration, and as it is held in the bladder Mg^{++} is added to it.

Figure 3 illustrates an experiment which lends support to this suggestion. The bladder of a crab that had been immersed in 100% sea water was drained and flushed with perfusion fluid in order to remove high concentrations of residual Mg^{++} . After flushing the bladder, the fluid was removed and the crab with the empty bladder was reimmersed in 100% sea water. Following a given period of immersion, the urine was removed from the same bladder and analyzed for Mg^{++} and Na^+ . As can be seen in Figure 3, 6 hours after reimmersion, the mean urine Mg^{++} concentration was low and the mean urine Na^+ concentration was high. As the immersion period increased and presumably the average period of urine retention increased, the mean urine concentration of Mg^{++} increased and the mean urine concentration of Na^+ decreased. For both urine Na^+ and Mg^{++} , the 92-96-hour group (mean) was greatly different from the 6-hour group ($P < 0.001$). It should be pointed out that urine samples taken 6 hours following bladder evacuation are also isosmotic with the blood. Thus, 15 crabs, which included 5 immersed in 100% sea water, 5 immersed in 158% sea water and 5 kept out of the water, had an osmotic U/B mean of 1.007 (S.D., 0.0176).

The possibility was considered that the empty bladder encouraged a rapid surge of fluid through renal organ and that insufficient time was allowed for Mg^{++} to concentrate in the urine before entering the bladder. As the bladder filled, the flow of urine through the labyrinth, for example, would be retarded and the subsequent urine entering the bladder would be relatively high in Mg^{++} . An experiment therefore was conducted showing that increases in Mg^{++} occur in the urine with time when the bladder is full.

Gross and Marshall (1960) gave evidence that *Pachygrapsus* does not lose urine when kept out of the water. The following preliminary experiment was conducted to demonstrate that fluid introduced into the bladder after artificial evacuation will be held in the bladder while the animal is kept out of the water. Urine from one bladder of the crab was emptied, flushed with an isosmotic solution colored with indigo carmine, emptied again and refilled. If there was no immediate sign of leakage due to injury of the nephropore, the dried animal was placed in a dry plastic container, the floor of which was covered with several layers of white absorbent tissue paper. In such a situation any loss of "urine" would stain the white paper. Of 20 animals thus tested using the following isosmotic solutions: (a) perfusion fluid for 24 hours (10 crabs); (b) NaCl for 3 hours (7 crabs) and (c) $MgCl_2$ for 3 hours (3 crabs) only one (NaCl) lost "urine" but this still had dye in the "urine" remaining in the bladder, indicating that only part of the introduced fluid leaked out. All other crabs retained sufficient color in the bladder fluid until the end of the experiment to have stained the white paper had fluid been lost. Still, after 24 hours the bladder fluid had lost considerable color, indicating absorption of the dye. Thus, such an experiment would be of little value if continued for more than one day. Nevertheless, the probability is high that isosmotic fluids introduced into an empty bladder will remain there for at least 24 hours if the crab is kept out of the water. It should also be noted that when dye is introduced

into one of the paired bladders, it does not appear in the other side, indicating that the bladders are isolated from each other.

Next, bladders of crabs removed from 100% sea water were evacuated, rinsed and filled with the above-described perfusion fluid containing 10 mM/l. of Mg^{++} . The animals were placed in dry containers and after selected periods the bladder fluid was sampled and analyzed for Mg^{++} . The bladder fluid of 10 animals so treated averaged 33.2 mM/l. (S.D., 11.0) for Mg^{++} 1–3 hours after introduction of the fluid, whereas the bladder fluid of 8 crabs averaged 64.5 mM/l. (S.D., 27.5) after 28–48 hours. These two groups are significantly different ($P < 0.01$) and only part of this difference could be caused by water withdrawal (Table I). Thus, urine Mg^{++} concentrates with time in a full bladder. This is interpreted to mean that the walls of the bladder transport Mg^{++} into the urine and prolonged retention of urine in the bladder results in the attainment of high Mg^{++} concentrations in the urine.

Evidence has been produced that *Pachygrapsus* does not lose urine when out of the water. On the other hand, when the bladder is emptied, it will readily fill even though the crab is not immersed. Substantial urine samples can be extracted from the bladders of most "dry" crabs 6 hours after bladder evacuation. Twenty-four hours after emptying, the bladders of crabs kept in dry situations seem as full as those of immersed crabs.

Figure 4 illustrates how crabs placed in dry containers with empty bladders (previously rinsed with isosmotic perfusion fluid) concentrate Mg^{++} in the urine with time at the expense of Na^+ . As shown for the immersion experiments, urine Mg^{++} increases with time after bladder evacuation, but Na^+ decreases with time.

Now if the period of time urine is held in the bladder dictates the concentration of urine Mg^{++} , then blocking the nephropore to prevent urine release should result in an increase in the Mg^{++} concentration of the urine. Thus, one of the paired nephropores of *Pachygrapsus* was blocked with epoxy cement and after the animal was immersed in 50% sea water for 24 hours, urine from both blocked and unblocked sides was extracted and analyzed for Mg^{++} . In every case (12) urine from the blocked bladder was higher in Mg^{++} than urine from the unblocked bladder, the mean ratio, blocked/unblocked being 2.63 (S.D., 1.19) which is significantly different from unity ($P < 0.001$).

Four lines of evidence have been presented indicating that the bladder of *Pachygrapsus* transports Mg^{++} from the blood into the urine, thus increasing the concentration of Mg^{++} in the urine with time as it is retained in the bladder far beyond that which could be caused by water withdrawal (Table I): (1) Crabs immersed with empty bladders show increased urine Mg^{++} concentrations with time; (2) when perfusion fluid is substituted for urine in the bladder, the Mg^{++} concentration of the bladder fluid increases with the period the crabs are kept out of the water; (3) when crabs with emptied bladders are kept out of the water, fluid low in Mg^{++} fills the bladder, but with time the concentration of urine Mg^{++} increases; (4) when urine from blocked and unblocked bladders of the same immersed crab are compared, urine from the blocked side is higher in Mg^{++} than urine from the unblocked side.

Since the phenomenon illustrated in Figures 3 and 4 suggests a direct Mg^{++} - Na^+ exchange, isosmotic solutions of NaCl or $MgCl_2$ were substituted for urine in

bladders of crabs kept out of water. One bladder of each crab first was evacuated of urine, rinsed twice with isosmotic test solution and then filled with a volume of test solution which approximated the volume of urine removed. After the crab was kept for a given period in air, the test solution was removed from the bladder and analyzed for Na^+ and Mg^{++} . In this way Na^+ and Mg^{++} concentration changes could

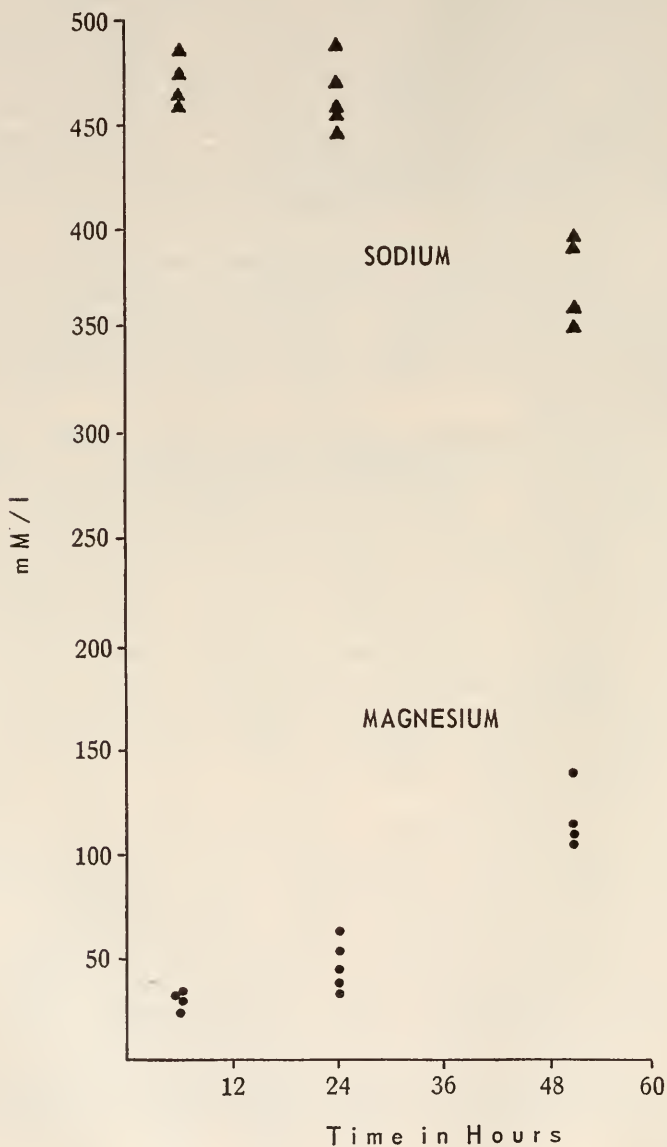


FIGURE 4. Decreases in urine Na^+ concentration accompanying increases in urine Mg^{++} concentration in crabs kept out of the water as a function of time after bladder evacuation. Triangles represent urine Na^+ ; circles urine Mg^{++} . Each point represents a single determination.

be measured in the bladder fluid and assuming constancy of bladder fluid volume, this information could give the relative number of Na^+ ions exchanged for Mg^{++} ions. Table II includes all cases of this experiment where there was no immediate indication of leakage from the nephropore due to injury and where there was sufficient concentration change of both ions to be measured quantitatively. Thus, it can be seen for both NaCl and MgCl_2 that whenever there was a gain in Mg^{++} concentration in the bladder fluid there was a loss in Na^+ concentration and *vice versa*. The

TABLE II
 *$\text{Na}^+-\text{Mg}^{++}$ exchange through bladder wall of *Pachygrapsus**

Bladder solution	Spec. no.	Na^+ change (mM/l.)	Mg^{++} change (mM/l.)	$\frac{\text{Na}^+ \text{ change}}{\text{Mg}^{++} \text{ change}}$	Time hrs.
560 mM/l. NaCl		—	+		
	1	116	82	1.42	1.0
	2	80	44	1.82	1.5
	3	100	55	1.82	2.0
	4	126	87	1.45	3.0
	5	80	31	2.58	3.0
	6	47	24	1.96	3.0
	7	64	36	1.78	3.0
	8	56	31	1.81	3.0
	9	81	69	1.17	18.0
	10	79	48	1.64	18.0
	11	96	72	1.33	18.0
	12	78	69	1.13	18.0
	13	48	34	1.41	18.0
	14	130	74	1.76	19.0
360 mM/l. MgCl_2		+	—		
	15	52	23	2.26	1.0
	16	265	218	1.22	1.0
	17	235	154	1.53	1.0
	18	218	127	1.72	1.0
	19	420	264	1.59	1.0
	20	362	233	1.55	1.0
	21	60	35	1.71	1.5
	22	322	221	1.46	2.0
	23	241	155	1.55	19.0
	24	358	219	1.63	19.0
Mean				1.64	
S.D.				0.33	

mean ratio, Na^+ concentration change/ Mg^{++} concentration change, was 1.64. Now, assuming no net anion movements, for every divalent Mg^{++} ion transported, two monovalent Na^+ ions should be exchanged. Chloride constitutes most of the urine anions because the urine for ten crabs removed from normal sea water had a mean osmotic concentration of 1040 mOsm/l. (S.D., 12.6) and a mean urine chloride of 516 mM/l. (S.D., 27.2). Green *et al.* (1959) stated that if $\text{Na}^+-\text{Mg}^{++}$ exchange occurred, the Na^+ change/ Mg^{++} change should be 2. However, the loss of two Na^+

ions for every Mg^{++} ion gained would reduce the osmotic concentration of the bladder fluid. Yet isosmotic NaCl solution introduced into empty bladders of 8 crabs remained essentially isosmotic for three hours when the animals were kept out of the water, the mean osmotic urine/blood value being 1.01 (S.D., 0.018). Since the Na^+ - Mg^{++} exchange would reduce the urine osmotic concentration, water must move to effect the isosmotic condition between blood and urine. Therefore, the observed Na^+ change/ Mg^{++} change should be related to the isotonic coefficients for NaCl and $MgCl_2$ which were empirically determined to be 1.8 and 2.8, respectively, at the initial test concentration (Table II). Therefore, the Mg^{++} concentration change $\times 2.8 = Na^+$ concentration change $\times 1.8$, or Na^+ concentration change/ Mg^{++} concentration change $= 2.8/1.8 = 1.56$, a value which closely approximates the observed value, 1.64 (Table II). This close agreement is interpreted as evidence that a direct Na^+ - Mg^{++} exchange can indeed take place. It also seems that such an exchange can take place in either direction across the membranes of the bladder. This in turn suggests relative impermeability of those membranes to chloride.

However, when isosmotic perfusion fluid was used instead of NaCl or $MgCl_2$, the mean Na^+ concentration change/ Mg^{++} concentration change for 6 crabs after 24 hours was only 1.15 (S.D., 0.14) which is significantly less than the mean 1.64 given in Table II ($P < 0.001$). The longer test period for perfusion fluid was necessary to permit a measurable cation change. A possible reason for these conflicting results will be given below.

The question now may be raised as to the dependence of Mg^{++} transport on Na^+ active transport. An attempt therefore was made to block active transport of Na^+ from the lumen of the bladder into the hemocoel by ouabain which is known to inhibit Na^+ transport (Judah and Ahmed, 1964). One bladder of the crab was drained of urine, rinsed with isosmotic perfusion fluid containing 10 mM/l. Mg^{++} and refilled with the same perfusion fluid containing 5×10^{-4} or 10^{-3} M ouabain. The crab then was placed in a dry container for 24 hours after which time the bladder was drained again and urine analyzed for Mg^{++} . The mean urine Mg^{++} concentration for 14 crabs thus treated was 120 mM/l. (S.D., 88.5). Even though low activity of the crabs indicated that the ouabain had diffused into the blood and was present on both sides of the bladder membrane, it obviously did not prevent accumulation of Mg^{++} in the bladder fluid.

The mean urine Na^+ concentration of 13 crabs after this treatment was 460 mM/l. (S.D., 27.4) which was not significantly different from the initial Na^+ concentration (483 mM/l.). However, the highest urine Mg^{++} concentrations were accompanied by the lowest Na^+ concentrations, so it is believed that either Na^+ movement, in this case, is a passive process or ouabain was ineffective in blocking the Na^+ transport mechanism in all cases. Nevertheless, there is no evidence that Mg^{++} secretion is coupled to the Na^+ transport mechanism, but there is further evidence that Mg^{++} accumulates in a full bladder with time. It might be that the Mg^{++} ion can exchange for any other cation, but since Na^+ is the dominant one, loss of Ca^{++} or K^+ from the urine in exchange for Mg^{++} could not be detected by the methods used in this investigation. Exploratory experiments where the bladder was filled with a perfusion fluid in which choline was substituted for Na^+ showed that Mg^{++} was concentrated in the bladder fluid after 24 hours. However, Na^+ was

also high in the bladder fluid and had obviously diffused from the blood down the steep gradient. Thus, it was not determined whether or not Mg^{++} was exchanged for choline.

In view of the above findings, there can be little doubt that Mg^{++} is concentrated in the urine by the bladder and that the Mg^{++} concentration is a function of the time urine is retained in the bladder. However, evidence was produced in the following experiments that the rate of Mg^{++} transport into the bladder is higher when the crab is in hypersaline water than when in normal sea water. That is, the amount of Mg^{++} entering the bladder 6 hours following evacuation is greater in crabs immersed in hypersaline water than those immersed in normal sea water.

In Group One (10 animals), the bladder of the crab was drained and flushed with isosmotic perfusion fluid, then drained again. The crab was reimmersed in 100% sea water and after 6 hours, urine from the same bladder was sampled for Mg^{++} analysis.

In Group Two (17 animals), the crab was first immersed in 158% sea water for 18–24 hours; the bladder was drained, rinsed with isosmotic perfusion fluid containing about 15 mM/l. Mg^{++} and reimmersed in 158% sea water for an additional 6 hours. After this period the urine was completely drained from the same bladder for Mg^{++} analysis.

In Group Three (20 animals), the crab was first immersed in 158% sea water for 18 hours; the bladder was drained, rinsed with isosmotic perfusion fluid containing about 15 mM/l. Mg^{++} and reimmersed in 158% Mg^{++} -free sea water for an additional 6 hours. The urine was then extracted for Mg^{++} analysis.

Thus, the 6-hour urine sample for crabs immersed in 100% sea water (Group One) averaged 54.5 mM/l. (S.D., 20.1), whereas for crabs immersed in 158% sea water (Group Two) the mean urine Mg^{++} was 113 mM/l. (S.D., 70.0). A second 6-hour sample was taken from Group Two (*i.e.*, 12 hours after rinsing of the bladder and reimmersion in 158% sea water) and the mean urine Mg^{++} then was 100 mM/l. (S.D., 48.5), indicating that the difference between 6-hour urine Mg^{++} in 100% and 158% sea water treatments is not merely a matter of residual Mg^{++} in the bladder of crabs immersed in 158% sea water for 18 hours. The mean urine Mg^{++} for Group Three which had been immersed for 6 hours in 158% Mg^{++} -free sea water was 120 mM/l. (S.D., 63.0). This mean as well as those for Group Two are significantly larger than the mean for Group One ($P < 0.01$).

Inulin U/B values (Table I) indicate that water withdrawal from urine is no greater for crabs immersed in 158% sea water than for those immersed in 100% sea water. Therefore, the different urine Mg^{++} concentrations produced during the 6-hour period by crabs in the two salinities cannot be explained on the basis of water withdrawal.

Inasmuch as the 6-hour urine sample for crabs immersed in 158% Mg^{++} -free sea water (Group Three) was equally as high in Mg^{++} as that of crabs immersed in 158% sea water containing high Mg^{++} , there is evidence that Mg^{++} transport from blood to urine is independent of the Mg^{++} concentration in the external medium and in turn independent of the Mg^{++} influx from the external medium to crab.

The higher concentrations of urine Mg^{++} observed above in crabs immersed for 6 hours in hypersalinity over those immersed for 6 hours in 100% sea water may indicate that: (1) the rate of Mg^{++} transport from blood into urine is higher

when the crab is immersed in hypersaline water than when it is immersed in normal sea water; (2) the rate of Mg^{++} transport is constant, but the volume of primary urine formed is smaller after 6 hours in 158% sea water than after 6 hours in 100% sea water, thus effecting a higher concentration of Mg^{++} in the urine while accumulating the same amount of Mg^{++} , and (3) there is reduced primary urine accompanied by increased Mg^{++} transport for crabs immersed in hypersalinities compared to those in normal salinity. If primary urine were formed by filtration, its rate of formation would be expected to be slower when the crab was in hypersalinities than when in normal salinity. Lockwood (1962) discusses the possibility of renal filtration among crustaceans in general. Kirschner and Wagner (1965) produce evidence of filtration in a fresh-water crayfish. To date, no reliable values have been obtained on the rate of primary urine production in *Pachygrapsus* for any treatment. However, evidence will be produced below that there is actually a

TABLE III
Elements influencing the concentration of Mg^{++} in urine

	Group A			Group B			Group C		
	50% sea water with 65 mM/l. of Mg^{++} for 18 hours to 100% sea water (6 hours)			158% sea water with 59 mM/l. of Mg^{++} for 18 hours to 100% sea water (6 hours)			158% sea water with 82 mM/l. of Mg^{++} for 24 hours to 158% Mg^{++} -free sea water (6 hours)		
	No.	Mean	S.D.	No.	Mean	S.D.	No.	Mean	S.D.
Urine Mg^{++} (mM/l.)	8	29.9	13.2	14	60.6	39.7	11	123.8	66.6
Blood Mg^{++} (mM/l.)	11	12.4	2.11	15	14.8	5.68	12	14.5	1.75
Blood osmotic concentration (% sea water)	12	93.7	4.60	15	115.5	2.51	12	135.0	6.97

higher rate of Mg^{++} transport for crabs in hypersaline water than for those immersed in 100% sea water.

Assuming for the moment such an increase in Mg^{++} transport does occur, then any of the following or combination of the following could be responsible for triggering the accelerated rate of such transport from blood into the bladder: (1) direction of passive water flux between animal and medium; (2) osmotic concentration of the external medium; (3) osmotic concentration of the blood, and (4) Mg^{++} concentration in the blood. Mg^{++} concentration in the medium and Mg^{++} influx already have been ruled out as triggering stimuli.

The experiment summarized in Table III was designed to test the direction of passive water flux and blood osmotic concentration as factors for controlling the rate of Mg^{++} transport when the blood Mg^{++} and osmotic concentration of the medium were held constant. Thus, Group A was immersed for 18 hours in a medium equivalent to 50% sea water in osmotic concentration, but containing 65 mM/l. of Mg^{++} which is about twice that present in 50% natural sea water. After 18 hours immersion one bladder of the crab was emptied, rinsed with isosmotic

perfusion fluid and emptied again. The crab then was immersed in 100% sea water for a period of 6 hours, after which time urine was removed from the same bladder for Mg^{++} analysis.

Group B was immersed for 18 hours in a medium equivalent to 158% sea water in osmotic concentration but containing 59 mM/l. of Mg^{++} which is about that found in 100% natural sea water and comparable to the concentration of Mg^{++} in the medium for Group A (above). After 18 hours, one bladder of the crab was emptied, rinsed with isosmotic perfusion fluid, emptied again and reimmersed in 100% sea water for 6 hours. After this period, the same bladder was drained and the urine analyzed for Mg^{++} .

For the second step of this experiment, that is, immersion in 100% sea water, the blood of Group A was osmotically less concentrated than the medium and the blood of Group B was osmotically more concentrated than the medium (Table III). Thus, with respect to the direction of passive water flux, Group A was simulating hypo-regulation (passive water efflux) and Group B hyper-regulation (passive water influx) which normally, when observed in crabs in high and low salinities, are accompanied by high and low urine Mg^{++} concentrations, respectively. If, then, the direction of passive water flux were a major factor in triggering the acceleration of Mg^{++} transport, Group A should have produced a more concentrated urine Mg^{++} during the 6-hour period than Group B. As can be seen in Table III, however, Group B produced the more concentrated urine Mg^{++} ($P < 0.02$). Since the external medium was the same for both groups, the cue for Group B to produce high urine Mg^{++} could not have come from the external medium during the 6-hour period. Furthermore, because the passive water flux in Group B was inward and in Group A was outward, the volume of primary urine formed should be higher in Group B than in Group A, again, assuming a filtration process. It is interpreted that the rate of Mg^{++} transport was indeed responsible for the difference between Groups A and B with respect to urine Mg^{++} concentration, a condition caused by the preliminary treatment in the dilute and concentrated sea water. There is evidence, then, that the rate of Mg^{++} transport is elevated when the salinity of the external medium is increased. Although the experiment was designed to maintain constant concentrations of blood Mg^{++} for both groups, it can be seen that the mean blood Mg^{++} concentration of Group B was higher than that of Group A ($P < 0.02$). Also, the blood osmotic concentration of Group B was, by design, higher than that of Group A ($P < 0.001$). Therefore, high blood Mg^{++} and/or osmotic concentrations possibly triggered the acceleration of Mg^{++} transport.

Group C was treated as follows in an attempt to lower the blood Mg^{++} concentration to that of Group B, but to elevate the blood osmotic concentration above that of Group B. First, the crab was immersed in 158% sea water (82 mM/l. of Mg^{++}) for 18 hours; (2) then the bladder was drained and rinsed with isosmotic perfusion fluid; (3) the crab was reimmersed in 158% sea water for an additional 6 hours when the bladder was again drained, rinsed as before, and (4) the crab was reimmersed in 158% Mg^{++} -free sea water for 6 hours after which the urine was sampled for Mg^{++} analysis. In the above procedure initial exposure to 158% sea water containing natural amounts of Mg^{++} was for the purpose of elevating the blood osmotic concentration by prolonged exposure to hypersaline water; transfer to 158% Mg^{++} -free sea water for the brief period was to maintain high blood osmotic

concentrations, but to reduce the blood Mg^{++} concentration to approximately the level achieved in Group B.

As seen in Table III the mean urine Mg^{++} concentration of Group C is higher than that of Group B ($P < 0.02$); mean blood Mg^{++} concentrations for the two groups are not significantly different, but the mean blood osmotic concentration of Group C is considerably greater than that of Group B ($P < 0.001$). It might seem that high blood osmotic concentration triggers the acceleration of Mg^{++} transport. However, values in Table III are terminal and while the mean blood Mg^{++} values for Groups B and C were essentially the same, it is likely that they changed during the 6-hour period when the sampled urine was being formed. On the other hand, as pointed out above, 6-hour urine samples from crabs immersed in 158% sea water have the same concentrations of Mg^{++} whether or not Mg^{++} is present in the medium. There does seem to be some evidence that the osmotic concentration of the blood gives the cue for setting the rate of Mg^{++} transport into the bladder.

Attempts were made to lower blood Mg^{++} further while maintaining high blood osmotic concentrations by prolonged immersion in Mg^{++} -free, hypersaline water. However, individual responses to such treatment were too variable (probably due to different rates of blood Mg^{++} depletion) to permit adequate resolution. On the other hand, blood Mg^{++} concentrations could be elevated while maintaining the blood osmotic concentration constant. Thus, crabs removed from 100% sea water were injected with 0.5 ml. of isosmotic $MgCl_2$ (360 mM/l.) after the bladder was evacuated and rinsed with isosmotic perfusion fluid. The crabs were reimmersed in 100% sea water for 6 hours after which the urine and blood were sampled for Mg^{++} analysis. Thus, the mean blood Mg^{++} (19 cases) was 20.6 mM/l. (S.D., 6.55) and the mean urine Mg^{++} (12 cases) was 158 mM/l. (S.D., 31.4). The mean blood Mg^{++} was significantly higher ($P < 0.001$) than the mean value 10.0 mM/l. reported for normal crabs immersed in 100% sea water by Gross (1959); the mean (6-hour) urine Mg^{++} was significantly higher than the 6-hour urine Mg^{++} (54.5 mM/l.) reported above for crabs with empty bladders immersed in 100% sea water ($P < 0.001$). Six crabs treated in the same manner but injected with 0.5 ml. of isosmotic perfusion fluid rather than $MgCl_2$ had a mean urine Mg^{++} of 52.8 mM/l. (S.D., 20.6) which was also significantly less than the value for the Mg^{++} treatment ($P < 0.001$). Since the injected $MgCl_2$ was isosmotic with the blood, the critical factor in elevating the urine Mg^{++} appears to be the concentration of blood Mg^{++} . There is evidence, therefore, that the rate of Mg^{++} transport into the bladder is influenced by the concentration of Mg^{++} in the blood.

It is concluded that the concentration of urine Mg^{++} in *Pachygrapsus* is determined by: (1) the length of time urine is retained in the bladder, and (2) the rate of transport for Mg^{++} into the bladder. Factors which influence the rate of Mg^{++} transport are: (a) the concentration of blood Mg^{++} , and (b) possibly the osmotic concentration of the blood.

There is no evidence that osmotic or Mg^{++} concentrations of the medium directly influence the rate of Mg^{++} transport. Neither is there evidence that the Mg^{++} flux or the direction of passive water flux directly influences the rate of Mg^{++} transport.

DISCUSSION

There is now convincing evidence that urine first entering the bladder of *Pachygrapsus* has a low concentration of Mg^{++} but a high concentration of Na^+ . In time

the urine Mg^{++} concentration increases and the urine Na^+ concentration decreases (Figs. 2, 3 and 4). This probably is accomplished, in part, by a direct Na^+ - Mg^{++} exchange across the bladder membranes.

The mean Na^+ concentration change/ Mg^{++} concentration change, 1.64, observed in solutions of $NaCl$ or $MgCl_2$ introduced into bladders of crabs kept out of the water (Table II) is compatible with this scheme. Yet, as shown above, when isosmotic perfusion fluid was used instead of $NaCl$ or $MgCl_2$ the ratio was only 1.15, a value that approximates the ratio derived from differences in means for Na^+ and Mg^{++} that occur with time in Figure 3. This conflict may be related to the large Na^+ gradient between blood and urine created by the introduction of pure solutions of $NaCl$ or $MgCl_2$ into the bladder. If the membranes were permeable to Na^+ and Mg^{++} but far less permeable to Cl^- , the rapid diffusion of Na^+ down the gradient across the membranes would necessitate a rapid Mg^{++} exchange because of the low Cl^- permeability. On the other hand, with the slow transport of Mg^{++} that normally occurs into the urine, the probability would be higher that a given Mg^{++} ion could be accompanied by Cl^- ions, thus reducing the necessity of Na^+ exchange for electro-chemical balance and therefore reducing the value for Na^+ concentration change/ Mg^{++} concentration change.

Should Cl^- move with Mg^{++} , then an osmotic increase would be caused in the urine, and this would result in an influx of water. Yet, the efflux of exchanged Na^+ would reduce the osmotic concentration of the urine, thus effecting an efflux of water. Since inulin U/B values (Table I) are not less than unity, it is unlikely that net increases in bladder fluid are caused by the inward movement of Cl^- with the transported Mg^{++} .

These data then suggest that during the normal processing of urine in the bladder, there is a direct exchange of Na^+ for the Mg^{++} that is secreted into the bladder, but also, there is some movement of Cl^- with the Mg^{++} , but not in sufficient amounts to cause a net gain of water in the bladder.

Riegel and Lockwood (1961) observed increases in the urine Mg^{++} concentration of *Carcinus* and decreases in urine Na^+ concentration with time as the crab was kept out of water. The increase in Mg^{++} concentration was attributed to Mg^{++} secretion and water withdrawal. However, these authors discounted a direct Na^+ - Mg^{++} exchange mechanism because during the test period (*e.g.*, 96 hours) the fall in urine Na^+ concentration (90 mM/l.) seemed too small to account for the rise in urine Mg^{++} concentration (103 mM/l.) on the basis of electro-chemical balance. Now, this might suggest that a direct Na^+ - Mg^{++} exchange was not the only process involved, but it does not rule out such a mechanism, for as pointed out above, electro-chemical balance could be achieved both by Na^+ - Mg^{++} exchange and Cl^- movement. Besides, Riegel and Lockwood point out that there is water withdrawal from the urine and in such a situation, the movement of Na^+ from urine to blood would be partially obscured by water withdrawal which would increase the concentration in the urine. On the other hand, the apparent movement of Mg^{++} from the blood to the urine would be exaggerated by water withdrawal increasing the Mg^{++} concentration. In end effect, withdrawal of water would reduce the ratio, Na^+ concentration change/ Mg^{++} concentration change, below that anticipated.

The wide range of urine Mg^{++} concentrations observed in *Pachygrapsus* (Fig. 1) can be explained largely by the fluctuations of concentration occurring in individual

crabs (Fig. 2) which as indicated above probably reflect the periods of bladder evacuation.

A distinction should be made between the concentration of urine Mg^{++} and the actual net excretion of Mg^{++} ; the rate of Mg^{++} transport into the bladder which as indicated above can be varied to meet the load, would, of course, influence both of these, but where there is a prolonged retention of urine in the bladder (e.g., in a crab immersed in hypersaline water), resulting in high urine Mg^{++} , the steep Mg^{++} gradient between blood and urine would likely counteract the effect of accelerated transport. There is no evidence of a good correlation between the ability of a crab to concentrate Mg^{++} in its urine and its ability to regulate Mg^{++} in the blood (Gross, 1964). Gross and Marshall (1960) produced evidence that *Pachygrapsus* loses more Mg^{++} when immersed in 50% sea water than when immersed in 150% sea water even though the urine Mg^{++} of crabs in the dilute medium was only one-sixth the concentration of that for the crabs in hypersaline media. This is interpreted to mean that although the active rate of transport for Mg^{++} into the bladder may have been less for crabs in the dilute medium than in a hypersaline medium, rapid water influxes in the former precluded retention of urine in the bladder, permitting no time for the buildup of a Mg^{++} gradient, thus resulting in less diffusion of Mg^{++} from the urine back to the blood and consequently a greater net transport of Mg^{++} into the urine and to the outside.

It has been shown for *Carcinus* (Webb, 1940) and for *Cancer* (Gross, 1964) that increased Mg^{++} in the medium is reflected in higher urine Mg^{++} concentrations. Such was not shown for *Pachygrapsus* by Gross and Marshall (1960) even though the blood Mg^{++} concentration was elevated by the treatment. It is apparent now that *Pachygrapsus* retains urine in its bladder for a period during which time the urine Mg^{++} concentration is built up (Figs. 2 and 3). Such a phenomenon would shroud the effect of accelerated transport of Mg^{++} if the experiment were initiated on crabs with full bladders. Thus, a crab immersed in hypersaline Mg^{++} -free sea water will appear to concentrate urine Mg^{++} as if the ion were present in high concentrations in the external medium. In this situation Mg^{++} will continue to be pumped into a full bladder probably already containing a high concentration of Mg^{++} . If urine is not evacuated, the Mg^{++} concentration will elevate to a maximum level determined by the osmotic concentration of the isosmotic blood and urine and probably by the magnitude of the Mg^{++} gradient between blood and urine, which, in turn, will depend on the rate of Mg^{++} transport into the urine. Until bladder evacuation occurs no Mg^{++} will be lost by this route and decreases in blood Mg^{++} caused by transport of this ion into the urine could be offset by diffusion of Mg^{++} from the urine back into the blood. Data in Table II show that Mg^{++} can move from urine to blood. Also, if the transport of Mg^{++} involves a direct exchange with Na^+ as the evidence above suggests, the Na^+ concentration gradient may also limit the concentration of Mg^{++} in the urine.

It was only by measuring the Mg^{++} concentration in urine first entering the bladder that the influence of blood Mg^{++} on the rate of Mg^{++} transport could be shown in the present investigation. In the cases of *Carcinus* and *Cancer* where high Mg^{++} concentrations in the medium are reflected in high urine Mg^{++} concentrations when crabs with full bladders are used (Webb, 1940; Gross, 1964), the urine probably is held only briefly in the bladder, no time being permitted to

elevate the Mg^{++} concentration and consequently not obscuring the influence of blood Mg^{++} on the urine concentration of this ion. Gross (1957) produced evidence that the exoskeleton of *Pachygrapsus* is less permeable than that of *Cancer*. Greater water fluxes would be expected in highly permeable animals which, in turn, would not hold urine in the bladder for long; the concentrations achieved for urine Mg^{++} would be expected to be low compared with a relatively impermeable animal. This invites measurement of water fluxes in an array of crabs to determine if the rate of water turnover is related to the maximum concentrations of Mg^{++} achieved in the urine.

Obviously, precise measurements of urine flow would allow quantitative evaluation of the assertions made here. However, meaningful values for urine flow and the consequent ion losses would have to be made under conditions where evacuation of urine from the nephropore was allowed to proceed in a natural manner. Gross and Marshall (1960) have calculated urine flow in *Pachygrapsus* in various salinities from mean urine Mg^{++} concentrations and mean Mg^{++} losses to the medium. Since average values were used, relationships between urine Mg^{++} concentrations that fluctuate in individuals (Fig. 2) and Mg^{++} losses could not be resolved.

In view of the evidence produced above, direct catheterization would, by draining the bladder, deprive it of its normal renal function and probably give spurious values, for urine flow and ion loss. Experiments designed to measure the natural flow of urine in *Pachygrapsus* are in progress, but reliable data have not yet been obtained.

The urinary bladder of *Pachygrapsus* clearly is more than an organ of storage. Although the anatomical details of the bladder are not described, exploratory studies reveal it to be a highly complex, lobed structure similar to those described for other brachyurans in the review by Balss (1944) where histological evidence suggests a secretory function of the bladder wall.

These studies were supported by National Science Foundation Grants, GB-1092 and GB-3969. We wish to express our gratitude to Prof. E. B. Edney for his critical reading of the manuscript and to Messrs. John Armstrong and Steven Peterson for their able technical assistance.

SUMMARY

1. The concentration of urine Mg^{++} in immersed specimens of *Pachygrapsus* is independent of the Mg^{++} influx as well as the concentration of Mg^{++} in the medium. It is, however, a function of the salinity of the medium.

2. Low U/B values for inulin indicate that water withdrawal has little effect in causing the high urine Mg^{++} concentrations and Mg^{++} U/B values observed in *Pachygrapsus*.

3. Repetitive samplings of urine from individual crabs immersed in 100% sea water reveal that the urine Mg^{++} concentration fluctuates with time, varying as much as three-fold in a single crab. This is not believed to be due to fluctuations in the Mg^{++} transport mechanism.

4. The wide range of urine Mg^{++} concentrations observed in the field can be explained chiefly on the basis of fluctuating urine concentrations in individuals rather than on large variations in the ability to concentrate Mg^{++} .

5. There is evidence that the membranes of the bladder transport Mg^{++} from blood to urine, and the concentration of Mg^{++} attained in the urine of *Pachygrapsus* depends on the length of time that urine is held in the bladder. Thus, hypo-regulating crabs immersed in hypersaline water having a small water influx will hold urine in the bladder sufficiently long to build up the Mg^{++} concentration. Hyper-regulating crabs in dilute sea water with a large water influx release urine too frequently to permit Mg^{++} buildup. This explains how the urine Mg^{++} concentration can be independent of the Mg^{++} concentration in the medium, but is a function of the salinity of the external medium.

6. Fluctuating urine Mg^{++} concentrations in crabs are believed to indicate periods of bladder evacuation, low Mg^{++} following evacuation and high Mg^{++} preceding evacuation.

7. There is evidence that when Mg^{++} is transported into the urine through the bladder wall, electro-chemical balance is achieved by direct exchange with Na^+ , but also by some movement of Cl^- with the Mg^{++} . Such a mechanism is compatible with the observed decreases in urine Na^+ concentration accompanying increases in urine Mg^{++} concentration.

8. Crabs treated with the Na^+ transport inhibitor ouabain can concentrate Mg^{++} in the urine. Thus, there is no evidence that Mg^{++} transport is coupled to the active transport of Na^+ .

9. Mg^{++} transport from blood to urine is more rapid when the crab is immersed in high salinities than when immersed in low salinities. The mechanism controlling the rate of Mg^{++} transport seems to be triggered directly by the Mg^{++} concentrations in the blood and possibly by the blood osmotic concentration.

10. The concentration of Mg^{++} attained in the urine of a crab does not necessarily indicate the relative ability to excrete Mg^{++} . It is suggested that permeability of the animal to water determines the rate of water turnover and therefore the rate of bladder evacuation. This, in turn, limits the period during which Mg^{++} can be accumulated in a given volume of urine.

11. Direct catheterization of *Pachygrapsus* would be expected to deprive the bladder of its normal renal function, thus giving spurious values for urine flow and ion losses.

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ESTIMATES OF POPULATION DENSITY AND DISPERSAL IN THE NATICID GASTROPOD, *POLINICES DUPLICATUS*, WITH A DISCUSSION OF COMPUTATIONAL METHODS¹

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This paper reports an attempt to use marking of individual snails and capture-recapture methods to assess population density and rates of dispersal in a littoral population of *Polinices duplicatus* at Barnstable Harbor, Cape Cod, Massachusetts. Earlier work by the present authors on the ecology of the infauna of the sand-flats in the Barnstable area (D. C. G.), and on the general biology of *Polinices* spp. (W. R. H.), had indicated both the importance and the difficulties of density estimates in populations of *P. duplicatus*.

Standard methods such as direct counting of quadrats are completely unsuitable, as preliminary surveys for the present work showed. Some of the difficulties are those which would arise with any moderately large-sized, and relatively widely dispersed animal capable of burrowing—others are peculiar to *Polinices*, and result from aspects of the behavior of “moon-snails.” For example, they can burrow deep into the substratum, can remain immobile for considerable periods, and may show marked tidal periodicity. Thus the present study showed that the number of animals visible at or near the surface of the sand at any one time is but a fraction of the total inhabiting the area.

However, it is important to attempt an accurate assessment of the population density in *Polinices*—and in other “ecologically difficult” species such as the horse-shoe crab, *Limulus*. In many areas of tidal flats around Cape Cod and elsewhere, such animals are among the important “terminal consumers” in the majority of infaunal associations of invertebrates. Further, the commercial importance of naticid species as pests of shell-fisheries has long been recognized.

Techniques of capture, marking, release and recapture have been used extensively in studies of birds and small mammals, and of insects such as Lepidoptera and tsetse-flies. Such methods were developed independently by Jackson (1933, 1937, 1939, 1948) working on tsetse-flies, and by Lincoln (1930) working on ducks. The former author, in association with R. A. Fisher, evolved a more sophisticated arithmetical treatment which allowed estimates to be made on populations of changing density. Subsequent work on Lepidoptera (Fisher and Ford, 1947; Dowdeswell, Fisher and Ford, 1940, 1949) utilized “trellis” arrays and allowed estimates of death-rates and emergence-rates. Some of the complications

¹ Supported in part by a grant from the U. S. National Institutes of Health, GM 11693.

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arising from differential behavior within the populations studied have been discussed by workers on small mammals (for example, Evans, 1949, on house mice) and on insects (for example, Ayre, 1962, on ant colonies). The significance of the different methods of analysis used on capture-recapture data has been considered by Schumacher and Eschmeyer (1943), Leslie (1952; see also Leslie and Chitty, 1951; Leslie, Chitty and Chitty, 1953), DeLury (1958), Turner (1960) and Andrewartha (1961). Treatments which can be used to estimate the bias and precision of the results obtained have also been developed (Bailey, 1951, 1952), and the methods applied to a variety of terrestrial vertebrates and insects. However, capture-recapture methods have not previously been applied to any extent in studies of marine benthic animals.

METHODS

Use of capture-recapture methods to assess population density is based on a number of assumptions, and this population density experiment on *Polinices duplicatus* was designed to satisfy as many of these as possible. Preliminary surveys and assessments of population density and dispersal by other methods made this feasible. The most important condition is that the marked animals after their release become homogeneously dispersed through the unmarked population before resampling. On the other hand, the simplest methods of calculation involve the assumption that resampling takes place immediately after release of marked animals (thus before the population is altered by births, deaths, immigration or emigration). Sampling only adult snails and using a time interval of 24 hours removes the complications due to births or deaths in a long-lived (4–7 years, Hunter, unpublished) animal like *Polinices*. The accuracy of population estimates is most greatly increased by having second and third recaptures at exactly equal intervals of time, and further increases in accuracy can be effected with further recaptures. However, the increased accuracy of ten recaptures over nine is not great, and considerations of effort to be expended made a six-day capture-recapture series optimal for the present work. A final assumption in this work is that marked individuals are identical in terms of life expectancy and behavior with unmarked individuals, and that the actual marks are permanent. The conditions of survival of individual snails and of marks are certainly satisfied for the six-day period, but a temporary disturbance of behavior resulting from the handling involved in marking was detected. This is discussed more fully below, and arithmetical procedures which circumvent the effects of this behavioral change on population assessments are set out.

The populations of *Polinices duplicatus* in Barnstable Harbor are of enormous extent, and a limited area for the capture-recapture experiment had to be set out. Its size had to be practical for collection of all snails sighted, that is of all the proportion of the population visible at or near the surface. On the other hand it had to support a sufficient number of snails both to avoid the complications of "patchy" distribution and to give the increased accuracy resulting from samples of 100 or more. Lastly, if the rate of capture, and the total area collected, could be adjusted so that the recaptured (marked) snails numbered between 5% and 25% of each sample subsequently captured, the accuracy of the population assessment would be relatively high.

In the early summer of 1962, direct counting of quadrats was carried out in several parts of Barnstable Harbor, and the numbers of *Polinices* were counted along tidal contours to find an area of relatively uniform density. The locality chosen was on the southern shore of Barnstable Harbor, about 400 meters east of the dredged channel into Maraspin Creek, on a relatively uniform substrate of muddy sand, at latitude $41^{\circ}42.6'N$, and longitude $70^{\circ}17.9' W$. The size of the area chosen to fit the conditions described above (*i.e.*, with sufficient population numbers, but small enough to make recapture practical) was 1600 square meters, being a square 40 m. by 40 m. The tidal range at this locality is 6.8 feet at neaps and 13.1 feet at springs. The experimental area, laid out normal to the littoral contours, was just covered at low water of neaps, and was totally exposed only during spring tides. The area was actually marked out as a central 20-m. square (400 sq. m.) within the main 40-m. square (1600 sq. m.) by eight permanent steel pegs. During each day's work a six-foot wooden stake was placed at each peg. The dispersal experiment discussed later was carried out around a single peg which was placed about 250 m. west of the main experimental area.

Since the whole process of capture, marking and redispersal took about four hours, and since it was planned to sample at every second low tide for six days (to maintain a constant interval of 24.8 hours between samples), the choice of dates was limited. The main series was run on August 8 through 13, 1962 (hereafter referred to as days I through VI), actual times of predicted low water ranging from 1215 to 1640 hours D.S.T. The tidal cycle in Barnstable Harbor is slightly modified from a single semi-diurnal pattern, and the afternoon range is less than the overnight.

On each day the eight stakes were placed about two hours before low water, and collecting of the sample of *Polinices duplicatus* was begun immediately. All collecting was done by the authors alone, in less than two feet of water and during the ebbing tide. The area of the main (larger) square was repeatedly traversed in a series of strips (about 1.6 m. wide), with a regular alternation of starting point between the two collectors. In fact, no bias occurred between them with regard to total numbers of animals, size of animals, or number of marks recaptured. Every snail sighted in the area was collected, until no sightings occurred over a 10–15-minute interval, and this always resulted in a sample of over 150 snails. The time taken for this collection varied somewhat with visibility (*i.e.*, weather and extent of wave action) and, under better conditions, occupied about 90 minutes. On one occasion (day IV, see below) conditions were so bad that collecting had to continue for about three hours (*i.e.*, including a period of the rising tide). The snails collected were stored in buckets of frequently changed shallow water. Sorting and marking were carried out on a small table set up in the water near the sample area. Sorting consisted of removal of undersized (< 2 cm.) specimens of *P. duplicatus* and all specimens of the closely related *Lunatia heros* which had been picked up accidentally. These "rejected" snails were dispersed some distance away. At this stage on days II through VI, all marks recaptured were counted, and the snails then given the appropriate mark for that day. The actual numbers at this stage were: I—142, II—132, III—145, IV—157, V—161, VI—136. Preliminary marking experiments had been carried out earlier in the summer on over 100 snails. Test marks on all parts of the shell of about 15 model dopes, nail polishes

and marking pens showed that seven types would survive for over three weeks even on the apex of the snail. To avoid any differential predation of marked snails, the mark in the density experiment was placed in the umbilicus of the shell which is not normally exposed in life, either to predators or to abrasion in the sand. In fact, of the specimens marked during the main series, at least three had clearly distinguishable marks when recovered eleven months later, and one after three years. More confidently than is usual in such experiments, it can be assumed that no

TABLE I
Data from density square

Day	I	II	III	IV	V	VI	VIF
Number captured	(174) 142	(141) 132	(164) 145	(177) 157	(185) 161	(183) 136	(420+x) 420
Unmarked	142	129	122	99	94	89	147
Single Marks	I (Pink)	3	19	15	21	8	36
	II (Red)		3	19	14	11	48
	III (Black)			22	24	10	45
	IV (Green)				3	9	46
	V (Orange)					1	35
Double Marks	I + II		1	1			0
	I + III				3	3	11
	II + III			1		1	2
	I + IV				1		5
	II + IV					1	10
	III + IV					2	15
	I + V						6
	II + V						3
	III + V					1	7
Triples					1 ^A		1
Number marked	142 Pink	132 Red	145 Black	157 Green	161 Orange		3 ^B

Notes: Numbers in brackets above second row are actual captures including *L. heros* and undersized specimens of *P. duplicatus*.

^A One triple: I, II and III.

^B Two triples: I, III and V; One triple: II, III and IV.

marks were abraded or otherwise lost during the six days of the principal observations.

The marking was always carried out in the same way. Lots of 10 snails each were removed from water in the storage buckets and caused to withdraw totally. Their shells were then dried with paper towels and the umbilical area cleaned with a little alcohol, care being taken to avoid the aperture and operculum of the shell. The alcohol-cleaned spot then received the day's mark, and was allowed to dry for a maximum of four minutes before the snails were returned to the storage buckets. Periodically, batches of 30-40 marked snails were randomly distributed through the dispersal area (*i.e.*, the inner 20-m. square). The rationale for this dispersal

will be discussed below. Thus, the snails were out of their habitat for 1.5–2 hours, but out of water only during the actual marking, for a maximum of eight minutes. The arithmetic procedures employed in assessing population density from the data thus collected will be discussed in the section on analysis and interpretation.

An experiment on rates of dispersal in *P. duplicatus* was carried out in an adjacent area during the last three days of the main capture-recapture series. A total of 393 marked snails was released around a single fixed point and their dispersal followed visually for 30 minutes. On two subsequent days concentric circles were staked out at 1, 2, 3, 4, 5, 7, and 10 meters from the fixed point, and the numbers of marked and unmarked specimens of *Polinices* within each annulus recorded. Two types of marks were used. Of the 393 marked snails, 105 were freshly marked in the field with a single black line on the apex, while the remaining 288 had been captured on the previous day, taken to the laboratory, marked with a black cross and released after 24 hours.

In all, 1237 snails were marked in these series of experiments. On the last day, after the regular sampling, as many snails as possible were captured over a period of hours in both the "density square" and the "dispersal circle." Of 737 snails marked and released in the density experiment, 273 snails bearing 339 marks were recaptured and taken to the laboratory where maximum shell diameter was measured. Of the 393 snails marked and released in the dispersal experiment, 156 were finally captured and measured. Almost a year later, three collections totaling 402 snails were made in the density area and two marked snails were collected. At the same time, but independently by Dr. Ralph I. Smith, a further marked snail was found about 400 m. away. Three years later, a total of 183 snails collected in the general area included one marked snail (from the main density experiment), which was found about 300 m. from the density square.

RESULTS

The capture-recapture data from the density experiment are set out in Table I above. The numbers in brackets in the column of total captures represent the actual numbers collected including undersized specimens of *Polinices duplicatus*, and specimens of the closely-related *Lunatia heros* (see Methods above). As can be seen, the experiment was relatively successful in terms of satisfying the several conditions for greater accuracy outlined above. For example, the average number captured and marked in the regular samples was 147 and, with certain exceptions, the numbers of each mark recaptured made up 7.7% to 19.3% of the samples. As noted above, there was no detectable bias between the two collectors in any respect. From the data, there is no indication that any individual animal exhibits an increased or decreased likelihood of capture. Certain data in Table I are significant in this respect. An excess of double marks in any group captured would indicate that there were individual snails more prone to capture. This can be tested in several ways. For example, if we consider the column for the regular captures of day VI, total captured snails were 136, of which 39 bore a single mark and 8 double marks. On that day the cumulative total of marked individuals at risk was 586 (by addition of the appropriate values for unmarked snails newly marked each day) and the corresponding number for double (and triple) marks at risk was 143. Thus the capture-ratio for all marks was 47/586 or 0.080 and for double

marks 8/143 or 0.056. Applied at several levels, these sorts of ratios do not indicate any group of snails more prone to capture. On the other hand, estimates could be also biased by some individuals being *less* readily captured. A crude test of this, although it involves a nearly circular argument, can be justified by the fact that *all* individuals captured on days I through V were marked. Using the line for the total captures of day VI (that is, VIF), the unmarked captured number 147, and several of the different estimates calculated below (and based on the entire day's collection) place the total snail population in the square at about 990 individuals. Assuming this to be correct, there would be 404 unmarked individuals at risk on day VI; thus the estimated capture-ratio for unmarked individuals was 147/404 or 0.36, while the ratio for all marked individuals was 273/586 or 0.47. This could demonstrate the absence of excessive bias resulting from individuals being less readily captured, *except for* the hypothetical extreme case where a significant proportion of the population remained undetected for more than six days. On the basis of observed behavior, this extreme hypothesis is unlikely. It is worth emphasizing again that it was totally impossible for the marks in the umbilicus to influence the collectors during the capture of expanded snails.

One source of bias was detected subjectively during the sampling, and is revealed in the data of Table I. The normal behavior of moon-snails is temporarily disturbed by the handling involved in capture and marking. After release in the inner dispersal square, such snails usually re-expand within eight minutes (all do within twenty minutes), and soon burrow deep into the substratum where they remain expanded but immobile for some time. Of course, in this expanded but buried state they are not liable to detection and capture. At first, it was known only that this period of immobility extended for more than 24 hours but less than 49.6 hours (or four tidal cycles). Then it was observed that, although snails marked on the previous day were not present in any numbers during the regular sampling, that is, during a 90-minute period *before* the time of low water), they became obvious among the many active snails on the surface during the two hours *after* the tide had begun to rise. In other words, recovery from marking trauma is complete in just over two tidal cycles, or about 25.5 hours. There is other evidence that a proportion of the population will always become more active immediately after inundation by a rising tide, and that this can involve a condition of temporary hyperthermia (Hunter and Apley, 1965). The effect of trauma is reflected in the number of marks recaptured after only one day (Table I). For example, the number of mark I captured on day II is relatively low. (For convenience, we can refer to R_{xy} , being the number of recaptures on day y , bearing a mark from day x .) Thus R_{12} , R_{23} , R_{45} and the regular R_{56} are all relatively low. On the other hand two collections do not show this effect: day IV and the final collection of day VI (VIF). Collecting on both these occasions extended into the first hours of the rising tide: on day IV because of bad weather conditions slowing the collecting rate, and later on day VI as a result of the deliberate collection of the large final sample over a period of hours. In several of the calculations below, the data of R_{12} , R_{23} , R_{45} and the regular R_{56} are rejected as biased, while R_{34} and the final R_{56} can be utilized, along with the other data where more than one day has elapsed between marking and recapture, such as R_{15} , R_{25} , R_{35} etc. Finally, the marks borne by the four snails recaptured with triple marks are detailed below

Table I, and have to be incorporated into the number of single and double marks recaptured in certain of the calculations below.

The data from the dispersal experiment are summarized in Table II. Although less successful than the density experiment, and hardly a complete measure of the possible rates of dispersal, a few significant facts emerge. In the first 30 minutes, 376 of the 393 individuals had expanded and righted, and > 200 were out of sight, having burrowed into the sand. The three specimens in the 2.0-4.0-m. annuli were known to have expanded and "sailed" with the longshore tidal current, but several had crawled > 1 m. and at least two had covered 1.75 m. Comparison of the sighting records (after two tides) and the final collections (after four) shows that line-marked specimens recovered more rapidly than the X-marked ones did from their

TABLE II
Data from dispersal experiment

Annuli in meters		0.-0.5	0.5-1.0	1.0-2.0	2.0-3.0	3.0-4.0	4.0-5.0	5.0-7.0	7.0-10.0	Totals
At release	{ Cross	288	0	0	0	0	0	0	0	288
	{ Line	105	0	0	0	0	0	0	0	105
	{ Unmarked	?	?	?	?	?	?	?	?	?
After 30 min. Sight records	{ Cross	(16*)approx.		18†	2†	1†	n.d.	n.d.	n.d.	137-187†
	{ Line	100-150†		?	?	?	n.d.	n.d.	n.d.	
	{ Unmarked	?		?	?	?	n.d.	n.d.	n.d.	
After 2 tides Sight records	{ Cross	127		2	0	0	0	0	0	129
	{ Line	4		5	8	1	2	0	0	20
	{ Unmarked	2		3	1	9	7	17	24	63
After 4 tides Collection	{ Cross	8	38	71	19	7	4	1	n.d.	148
	{ Line	1	0	2	4	3	3	1	n.d.	14
	{ Unmarked	0	2	2	5	7	13	16	n.d.	45

* These 16 snails were the only ones not yet moving.

† Combined marks.

n.d. = No data available.

prolonged and more drastic handling. The circumstances affecting the density experiment discussed above are not valid here since both the sighting records and the final collection were made during the first hours of the rising tide. These data estimate dispersal rather than absolute rates of movement, and some subjective assessments made during the density experiment are relevant. Rates of movement may be high (as much as 3-4 m. in 15 minutes). In the course of one tidal cycle, snails were observed to have left tracks equalling 7-8 m. However, tracks are rarely straight, and often elaborately looped, so that each individual snail tends to remain in the same general area. Observations such as these led to the proportions arranged, in the density experiment, for the larger (sampling) area and the inner (dispersal) area (40 m. by 40 m. enclosing 20 m. by 20 m.). The minimum width of the outer zone was thus 10 m. All marks appeared in the outer zone within two days, and by the sixth day considerable numbers of first-day marks were found

just within the outer edge of the larger square. Actually at least one specimen of every mark was found just within the edge of the outer square during the final collection of day VI (VIF).

ANALYSIS AND INTERPRETATION

The simplest possible estimate from capture-recapture data, involving a single recapture of a single previous mark, is the "maximum-likelihood" estimate given by the formula:

$$P_{xy} = \frac{N_y M_x}{R_{xy}}$$

where P_{xy} is an estimate of the population based on the recaptures on day y of individuals marked on day x , N_y is the total captured on day y , M_x is the number of individuals bearing a mark from day x which are "at risk" on day y , and R_{xy} is the number of recaptured marks as defined earlier. Both for this, and for other more complex estimates below, it is worth setting out the data in a trellis

TABLE III
Trellis: raw data transformed to single marks

Capture days:			I	II	III	IV	V	VI	VIF
Number of marked indiv. captured			0	3	23	58	67	47	273
Number of marks captured			0	3	24	60	73	55	339
Number marked	I	142	—	3	20	16	26	11	60
	II	132	—	—	4	21	15	13	64
	III	145	—	—	—	23	28	17	83
	IV	157	—	—	—	—	4	12	78
	V	161	—	—	—	—	—	2	54
Unmarked indiv. captured			142	129	122	99	94	89	147
Total indiv. captured			142	132	145	157	161	136	420

array (as used by Dowdeswell, Fisher and Ford, 1940, 1949, but in the reorientated and typographically simpler form used by Andrewartha, 1961, and others). In the trellis (Table III), the raw data are transformed to single marks, and there is consequently a slight loss of information. In the trellis each horizontal row represents the recaptures of one day's marking on successive days, and each vertical column the recaptures of the available marks on one day. Diagonals across the central part represent the recaptures after an interval of one day, of two days and so on. The column headed VIF gives the final *total* number of snails captured on day VI and includes the regular sampling (column VI). On the diagonal of recaptures after a lapsed time of one day appear the recapture figures (R_{12} , R_{23} , R_{45} and Regular R_{56}) which are rejected on behavioral grounds. All the other R_{xy} data are available for calculation by this simple maximum-likelihood formula, and there are thus 16 possible estimates of P_{xy} . The estimates of P_{16} , P_{26} , P_{36} etc. derived from the VIF totals are to be preferred (since based on larger samples) to the regular VIth day series. This leaves the 12 values of P_{xy} which are calculated and form the seventh column of Table IV. These population estimates range from 733.7 (P_{36}) to 1416.8 (P_{25}), with a mean of 1,018.4, and a standard deviation (s.d.)

of 222.4. It should be noted that this s.d. is a measure of the variance of this group of 12 results, not a measure of variance of the population estimates as such. Bailey (1951, 1952) has examined the precision of such estimates of P_{xy} and shows a measure of the variance to be:

$$\text{Var. } P_{xy} = \frac{(M_x)^2 N_y (N_y - R_{xy})}{(R_{xy})^3}$$

expressed in the terms used in the present paper. Values can be calculated for the data in Table IV: for example, for the estimates over two days (P_{24}) and over five days (P_{16}) the variance values are 40,172.5 and 14,114.8, respectively. The

TABLE IV
Maximum likelihood estimate of population, simple computation

Estimate	On mark	Capture day	N_y	M_x	R_{xy}	$\frac{N_y M_x}{R_{xy}}$
P_{13}	I	III	145	142	20	1029.5
P_{14}	I	IV	157	142	16	1393.4
P_{24}	II	IV	157	132	21	986.9
P_{34}	III	IV	157	145	23	989.8
P_{15}	I	V	161	142	26	879.3
P_{25}	II	V	161	132	15	1416.8
P_{35}	III	V	161	145	28	833.8
P_{16}	I	VIF*	420	142	60	994.0
P_{26}	II	VIF*	420	132	64	866.3
P_{36}	III	VIF*	420	145	83	733.7
P_{46}	IV	VIF*	420	157	78	845.4
P_{56}	V	VIF*	420	161	54	1252.2

Mean 1018.4

* Final totals for VIth day used here.

root values, which can be considered as "standard errors" of the estimates, are 200.4 (for P_{24}) and 118.8 (P_{16}).

It is noted above that transforming the data to single marks results in a loss of information on the occurrence of multiple recaptures or double marks, which could yield an estimate of population size. The simple formula for a maximum-likelihood estimate can be adapted for double marks:

$$P_{(wx)y} = \frac{N_y M_{(wx)}}{R_{(wx)y}},$$

where $P_{(wx)y}$ is an estimate of the population based on the recaptures on day y of individuals with marks for both days w and x , and the other terms correspond. The numbers of double marks "at risk" on any day subsequent to the second marking can be derived from Table I, and are the terms for $M_{(wx)}$. The following values are available: $M_{(12)} = 3$, $M_{(13)} = 20$, $M_{(23)} = 4$, $M_{(14)} = 16$, $M_{(24)} = 21$, $M_{(34)} = 23$, $M_{(15)} = 26$, $M_{(25)} = 15$, $M_{(35)} = 28$, and $M_{(45)} = 4$. Rejecting the values for $R_{(12)3}$, $R_{(14)5}$ and $R_{(35)6}$ on the usual behavioral grounds, and omitting all calculations based on $R_{(wx)y} = 1$, then we have the 11 estimates of $P_{(wx)y}$ calculated and

given in Table V. These range from 560 to 2100, with a mean of 1091.2, and an s.d. of 484.1. Most individual variances of these estimates would be very large because of the low double recapture rates (low values of $R_{(wx)y}$), and they are not calculated here. The fact that these estimates of $P_{(wx)y}$ based on double marks are closely comparable to the values of P_{xy} calculated earlier is a further confirmation of a lack of "capture-prone" bias in individual snails.

A modification of the simple proportional formula was proposed by Bailey (1951, 1952) to reduce positive bias. He demonstrates (Bailey, 1951) that this modified estimate has an average relative bias that is more than order of magnitude lower

TABLE V

Maximum likelihood estimates of population, based only on "double"-marked individuals

Capture day	On mark	N_y	$M_{(wx)}$	$R_{(wx)}$	$\frac{N_y M_{(wx)}}{R_{(wx)}}$
V	I + III	161	20	4	805.0
VI	I + III	136	20	3	906.7
VI	III + IV	136	23	2	1564.0
VIF	I + III	420	20	13	646.2
VIF	II' + III	420	4	3	560.0
VIF	I' + IV	420	16	5	1344.0
VIF	II + IV	420	21	11	801.8
VIF	III + IV	420	23	16	603.8
VIF	I + V	420	26	8	1365.0
VIF	II + V	420	15	3	2100.0
VIF	III + V	420	28	9	1306.7

Mean = 1091.2

at certain levels of sample and population size and, expressed in our terms, has the form:

$$P_{xy} = \frac{M_x (N_y + 1)}{(R_{xy} + 1)}$$

Bailey (1951) also proposed a satisfactory approximation for the variance of this estimate of P_{xy} . The present data are unlikely to require such correction for positive bias. However, using the above formula on the data for single marks in Table III, yields estimates (not set out in the tables) with a mean of 986.6, an s.d. of 202.9, and 'standard errors' (using Bailey's approximation) of the order of 183.4 and 115.1.

A great deal of previous capture-recapture work, particularly with fish stocks, has utilized methods involving series of census carried out on stocks in which the marks were not distinguished as to date of origin. One of the best of such methods is that originated by Schumacher and Eschmeyer (1943) involving census weighted for sample size. The Schumacher-Eschmeyer method is discussed by DeLury (1958) and Turner (1960), and theoretical arguments given by DeLury, and practical reasons by Turner, for preferring it to a maximum-likelihood method, in certain cases. In general terms, it appears that a maximum-likelihood estimate is

still most efficient if sampling is truly random, and if random dispersal of marks is complete by the time of re-sampling, but that if sampling is biased for any reason a method involving weighting for sample size is to be preferred. The data on *Polinices duplicatus* can be used in a Schumacher-Eschmeyer computation. The general formula used is:

$$P(\text{Schumacher-Eschmeyer estimate}) = \frac{\sum N_d M_d^2}{\sum R_d M_d}$$

when M_d is the total number of marked *individuals* at risk on day d , N_d is the number captured on day d , R_d is the number of recaptured marked *individuals* in N_d . We are concerned here with numbers of individuals recaptured rather than number of marks, and it is necessary to use for M_d the cumulative totals for each day of all the marked individuals at risk. These can be derived from the sums of previously unmarked snails collected for marking (listed in column 2 of Table I). Thus we have as values for M_d on day II—142, III—271, IV—393, V—492 and VI—586. These values are used along with those for N_d and R_d in Table VI.

TABLE VI
Serial census method for Schumacher-Eschmeyer estimate of population

Day	M_d	N_d	R_d	$(R_d M_d)$	$(N_d M_d^2)$
dII	142	132	3	426	2,661,648
dIII	271	145	23	6,233	10,648,945
dIV	393	157	58	22,794	24,248,493
dV	492	161	67	32,964	38,972,304
dVIF*	586	420	273	159,978	144,226,320
				$\sum R_d M_d$ = 222,395	$\sum N_d M_d^2$ = 220,757,710

$$\therefore \hat{P} = 992.6$$

* Final totals for VIth day only used here.

The estimate of P derived by this method is 992.6. In spite of the fact that considerable information is lost by treating all marks as one type of mark, this Schumacher-Eschmeyer estimate is remarkably close to the mean of the maximum-likelihood estimates produced by Bailey's modified formula.

It is probable that the most complete utilization of capture-recapture data of the present sort is provided by a series of computations such as were developed by C. H. N. Jackson in his work on tsetse-fly populations in association with R. A. Fisher (Jackson, 1933, 1937, 1939, 1948; see also Fisher and Ford, 1947; Dowdeswell, Fisher and Ford, 1940, 1949). The theoretical bases of these methods are discussed by Bailey (1951) and by Leslie (1952), and simplified examples of the computations set out by Dowdeswell, Fisher and Ford (1940) and by Andrewartha (1961). The present data on *Polinices duplicatus* can be used both in Jackson's *positive* method, involving recapture on a number of occasions after one marking, and in Jackson's *negative* method, involving a single recapture of marks made on a series of occasions. Table VII is a trellis array of the values of R_{xy} to be used,

TABLE VII

*Trellis: selected recapture data as used in Jackson computations,
with calculated relative frequencies*

Capture days:		III	IV	V	VIF (total)	
Number marked	I	142	20 (9.7)	16 (7.2)	26 (11.4)	60 (10.1)
	II	132		21 (10.1)	15 (7.1)	64 (11.5)
	III	145		23 (10.1)	28 (12.0)	83 (13.6)
	IV	157				78 (11.8)
	V	161				54 (8.0)
Total individuals captured:		145	157	161	420	

Note: the numbers in brackets are relative recapture frequencies (f_{xy}), as defined in the text.

omitting the usual ones on behavioral grounds, and using only the total captures for day VI (VIF). These values of R_{xy} are first converted to relative recapture frequencies (f_{xy}) which correspond to what the recaptures would have been if 100 had been marked and 100 captured on each successive date. These are derived by:

$$f_{xy} = R_{xy} \times \frac{100}{M_x} \times \frac{100}{N_y}$$

We have twelve available values (for f_{13} , f_{14} , f_{24} , f_{34} , f_{15} , f_{25} , f_{35} , f_{16} , f_{26} , f_{36} , f_{46} and f_{56}) and these appear in brackets below the corresponding recapture figures (R_{xy}) in the trellis of Table VII. Each vertical column with three or more values of f_{xy} gives the number recaptured on one day for an estimate by Jackson's negative method; each horizontal row with three or more values of f_{xy} represents the subsequent recaptures of one set of marks and provides one estimate by Jackson's positive method. Thus we have six possible estimates, three by each method. We have first to calculate values of z and z^* , which are weighted ratios of relative recaptures (z for each positive, and z^* for each negative computation). Examples of these weighted ratios are:

$$z = \frac{f_{14} + f_{15} + f_{16}}{f_{13} + f_{14} + f_{15}} \quad (\text{positive method}), \text{ and}$$

$$z^* = \frac{f_{46} + f_{36} + f_{26} + f_{16}}{f_{56} + f_{46} + f_{36} + f_{26}} \quad (\text{negative method})$$

These are then employed in calculating a reciprocal value for the population estimate, which then requires multiplication by 100×100 to convert relative frequencies back to absolute numbers. Examples of the formulae are:

$$10^4(P)^{-1} = \frac{f_{13} + f_{14} + f_{15}}{z} - (f_{13} + f_{14}) \quad (\text{positive method}), \text{ and}$$

$$10^4(P)^{-1} = \frac{f_{56} + f_{46} + f_{36} + f_{26}}{z^*} - (f_{56} + f_{46} + f_{36}) \quad (\text{negative method}).$$

From the data of Table VII we have positive estimates of P of 908.3, 1720.9, and 1113.0, and negative estimates of 740.9, 1296.8, and 1054.4. The great value of the Jackson methods lies in their ability to deal with changing population densities, and these estimates above are extrapolations forwards or backwards and actually represent the population estimate on a specific day. The population of *Polinices* studied is thought to have been relatively stable over the short period of this experiment. Another advantage of the Jackson computations is that they are thus applicable in conditions where a longer delay is necessary after each release to allow marked animals to disperse and settle down. Obviously they will prove valuable with certain types of littoral populations but, in the present case, yield estimates no more valuable than the simpler ones, in spite of the more complete utilization of the data. Bailey (1951, 1952) has given methods for an approximate estimate of var. P for both positive and negative methods, but these are not computed for the present data. Leslie (1952; see also Leslie and Chitty, 1951; Leslie, Chitty and Chitty, 1953) has suggested slight elaborations of the Jackson methods, less general in application, but which can be more precise as a result of utilizing a still greater proportion of the information yielded by the data.

The above may serve to indicate which computational methods can be best applied to any future capture-recapture work on other marine benthic populations. Apart from behavioral considerations, the sample size, and the relative time-scales of sampling and of population life-cycle should be decisive. Turning to the results of the work on *Polinices duplicatus*, it seems clear that the true population level for the 1600 square meters at the time of the experiment lay close to the average values from maximum-likelihood estimates of 1018.4 (simple calculation), and of 986.6 (Bailey's modification), and from the Schumacher-Eschmeyer computation of 992.6. For comparative purposes, we can say that a close estimate is about 1,000 and, from the different variance values computed, that it was unlikely to lie outside 850–1150. Thus the density is probably about 6250 (between 5313 and 7188) per hectare, or about 2530 (between 2150 and 2900) per acre of intertidal flats.

The total area of Barnstable Harbor is approximately 3763 hectares, or 9298 acres. Of these 55% are occupied by salt marsh. The remainder consists of extensive sand flats (26%) dissected by permanent subtidal channels (19%). Throughout the sand flats (approximately 1672 hectares or 4132 acres) and channels (approximately 711 hectares or 1756 acres) there are populations of *Polinices duplicatus*. The population densities vary greatly within this area. For example, within the permanent channels densities are relatively low, while along the margins of these channels, on areas of higher organic content, the greatest densities for

the harbor may be reached. On the sand flats the greatest densities are found around the level chosen for the density square in the current study. Above and below this level the population densities are usually lower.

In the central areas of the harbor particularly, it is probable that the population densities of all moon-snails have been reduced since the time of this study (summer, 1962) by a human agency. Shellfish interests have supported extensive destruction of moon-snails and *Limulus* in subsequent summers. The effectiveness of this program has not been tested and is questionable.

One additional result should be mentioned here. At the conclusion of the VIth day's collections all 420 marked and unmarked specimens of *Polinices duplicatus* were taken to the laboratory where the maximum shell diameter was measured with a dial-caliper. The total number of marked individuals recaptured at that time was 273, and their size range was 18.8 to 53.9 mm. (mean = 29.27 mm., s.d. 7.012 mm.). Individuals with double and triple marks totalled 63, with a size range 19.5 to 45.6 mm. (mean = 28.98 mm., s.d. 5.710 mm.). Total unmarked captures (VIF) was 147, with a size range 17.6 to 52.2 mm. (mean 27.95 mm., s.d. 6.853 mm.). Thus there is little evidence of any size bias as regards chance of multiple recapture. In addition, the mean values lie within the range for other population samples from elsewhere in Barnstable Harbor (Hunter, unpublished). Further, it is worth noting that the "undersized" specimens of *P. duplicatus* which were "rejected" before marking had a mean size of 12.2 mm., and made up less than 10% of each sample collected and marked (that is, less than 1.5% of the estimated population). It is thus unlikely that their removal had any significant effect on the remaining adult population.

Finally, it should be pointed out that if this work is judged to be a relatively successful demonstration of capture-recapture methods applied to a marine benthic population, this results from two features. First, only two persons were involved in the field work, both of whom had considerable experience of the general biology of *Polinices*, including its behavior patterns. Secondly, because of this experience and preliminary survey work, it was possible to set up the density experiment with the optimum time interval, sample size, and recapture rate. It is clear that the application of these methods to a benthic population of totally unknown biology could show intolerable bias.

We wish to thank Mr. Homer P. Smith of the Marine Biological Laboratory, and members of the Supply Department staff, for making a truck available for the field work. We are indebted to Myra Russell Hunter, Martyn L. Apley, William Charles Blowers II, and Jay Shiro Tashiro for checking calculations, and to Anthony Williams for assistance preparing the estimate of area for Barnstable Harbor. We are particularly happy to thank Dr. Clark P. Read, who provided the opportunity to begin this work at Woods Hole.

SUMMARY

1. Capture-recapture methods were used to assess population density and dispersal in *Polinices duplicatus*.

2. Preliminary surveys indicated an area of 1600 sq. m. as optimal for density estimations. This yielded capture rates averaging 147 per day and the number of each mark recaptured usually made up 7.7% to 19.3% of each sample.

3. Various computational methods are applied to the data, and their values discussed. These include simple and modified maximum-likelihood estimates (with measures of their variance), a serial census method weighted for sample size, and the classical Jackson-Fisher extrapolations of relative recapture frequencies, both "positive" and "negative."

4. The two maximum-likelihood methods yield average population estimates of 1018.4 and 1091.2, and the sample-weighted census method yields a value of 992.6. With capture rates and mark frequencies as in this study on *Polinices*, these relatively simple calculations are judged adequate.

5. These estimates are equivalent to a population density for *Polinices duplicatus* of 6250 (5313-7188) per hectare, or 2530 (2150-2900) per acre.

6. Recommendations are made regarding the value of different computational methods, if capture-recapture methods are applied to other marine benthic populations. Approximate population density, sample size, and the relation of sampling-rate to population dynamics should be used to determine the appropriate procedure.

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DIURNAL PATTERNS OF METABOLIC VARIATIONS IN CHICK EMBRYOS¹

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One of the most obvious characteristics of living organisms is the dynamic nature of their physiological constitution. A commonly observed aspect of this dynamic nature is cyclical variation in various physiological processes. Many of these cyclical variations disappear under laboratory constant conditions, but others are very persistent even when the organism is removed from obvious environmental timers. These cyclical phenomena exhibit periodicities of many different lengths including approximate solar daily, lunar daily, semi-monthly, monthly, annual, and longer term ones (Harker, 1958, 1964; Brown, 1959a; Webb and Brown, 1959; Cloudsley-Thompson, 1961; Bünning, 1964; Sollberger, 1965; Aschoff, 1965).

Since the total physiology of any organism is so broadly related to these cyclical phenomena, it is of potentially great interest to consider the ontogeny of physiological periodicity. A basic question is whether an organism's physiology possesses cyclical qualities only in a relatively mature state or whether systematic variability can be demonstrated throughout the life-history of the organism. The former seems to be the case for some overt patterns of cyclical variability such as the human infant pulse rate and sleep-wakefulness cycles (Hellbrügge, 1960). However, since these functions become phased to obvious daily environmental fluctuations and are not observed in early developmental stages, it is of interest to seek periodicities which might exist under constant conditions and in earlier developmental stages.

Hiebel and Kayser (1949) were unable to detect a clear diurnal rhythm of movement of the chick embryo within the egg. They were measuring movements of the whole egg and were seeking a clear-cut nocturnal reduction in activity. Following hatching they could detect no diurnal rhythm in heat production unless they subjected the young chicks to alternating light and dark schedules.

Aschoff and Meyer-Lohmann (1954), however, recorded motor activity of newly hatched chicks and by summing all activity in four-hour periods could show, under constant conditions, a clear rhythmic variation in activity present from the time of hatching. This rhythm had an average period of 25.4 hours. Hoffman (1957, 1959) has shown an activity rhythm of approximately 24 hours for newly hatched lizards. Petren and Sollberger (1953) found that rhythmic fluctuations in liver glycogen content were already established in very young chickens (measurements made on days 5-6, 12-13, and 36-37 post-hatching). Further studies showed that a periodic fluctuation in glycogen content was already present on the

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first day after hatching and that a rhythm of somewhat different character could be demonstrated even if the newly hatched chicks were starved. In other experiments, freshly laid eggs were stored and incubated in total darkness until the twentieth day of incubation when liver glycogen determinations were made. A clear daily rhythm with a major peak and a minor peak of glycogen content was demonstrated. Therefore, the diurnal liver glycogen rhythm is well established before the time of hatching even under constant conditions.

These data relate, however, to rather advanced stages of development. One study carried out in earlier stages of development was the measurement of metabolic variations by Barnwell (1960). He was able to detect a significant mean solar daily fluctuation in rate of oxygen consumption. By measuring oxygen consumption under constant conditions, and treating data to correct for the increasing overall rate of oxygen consumption, such fluctuations were established to be present on the fifth through the eighth days of incubation (eggs were incubated at 4 PM and the day upon which incubation was begun was designated as day one).

Barnwell's work has been criticized (Heusner, 1963, 1965; Heusner and Zahnd, 1963) largely on the basis of suggested faults in his techniques. These criticisms appear to have originated largely from misinterpretations of experimental procedures and statistical techniques used by Barnwell, including underestimation of the sensitivity of his respirometers, and have been dealt with more extensively elsewhere (Johnson, 1965).

The presence of a mean daily cycle of variation in rate of oxygen consumption during these stages of development raises some new questions. It must be determined whether this periodicity has a definite pattern of ontogeny. The properties of metabolic periodicity in a developing organism should be compared with some well-known properties of other rhythmic systems. In order to make meaningful comparison, a consistent statistical treatment must be developed. Once such a treatment is available, questions about ontogeny of periodicity, seasonal cycle form differences, and temperature relationships should be answerable.

METHODS AND MATERIALS

Fertile White Leghorn eggs were obtained from a local commercial supplier and stored in a cold chamber at approximately 10° C. until used (some were incubated directly). Before incubation the eggs were allowed to warm to room temperature. Incubation was initiated at 9 PM (CST) for all experiments described here unless stated otherwise. The incubator was a forced draft model provided with water pans and set at 38° C.

Respiration was measured using Brown automatic recording respirometers (Brown, 1954, 1957). The six respirometer units were constructed at the beginning of the study.

In operation the sealed respirometer chamber was evacuated to produce an internal pressure of 28.7 inches Hg which then remained constant throughout each recording period. Temperature was controlled at 38° C. by a 55-gallon circulating water bath which surrounded the respirometer chamber, or barostat. A mercury switch and relay controlled a 450-watt immersion heater suspended in the bath. Water temperature cycled within a range of $\pm 0.05^\circ$ C. The experiments were conducted in a photographic darkroom in which the only light source was a single

ceiling fixture controlled through a voltage-regulator. The intensity of illumination reaching the interior of an uncovered barostat was less than one foot candle. With the glass cover in place, the respirometer in the interior was provided with a substantially lower level of constant dim light.

The respirometer flask itself was modified as described by Barnwell (1960), with minor changes. The support frame which held the egg in place was a cylinder produced by cutting a section out of a glass vial. The cylinder was perforated at several points to permit free passage of air under and around the egg. The egg was set on the support frame with the air chamber uppermost. A KOH solution in the bottom of the flask was the CO₂ absorbent and also served to meet humidity requirements. A single egg was suspended in the diver below the recorder and oxygen consumption values were calculated in ml. consumed per egg per hour.

Control experiments for the apparatus were conducted at the beginning of the experimental period and at various times thereafter. Extended recordings of the

TABLE I
Comparison of rates of O₂-consumption as ml. of O₂/egg/hour at several stages of incubation as determined in various studies

Hrs. incub.	This study (spring)	I*	II	III	IV	V	VI	VII	VIII
72	0.283±0.055†	0.266±0.066	0.253±0.040					0.216	
96	0.445±0.105	0.427±0.110	0.381±0.100	0.34±0.11				0.333	
120	0.715±0.101	0.680±0.131	0.524±0.025	0.44±0.05			0.612	0.628	
144	1.071±0.199	0.826±0.178	0.831±0.134	0.89±0.07	0.91±0.06		1.542	0.922	0.883
168	1.752±0.162	1.423±0.191	1.421±0.223		1.19±0.14	1.19	2.075	1.775	1.168
192	2.221±0.145	1.922±0.173	1.671±0.173	1.23±0.26	1.51±0.03	1.52	2.262	2.428	1.835

* I Barnwell, 1960; II Romanoff, 1941a; III McLimans, Siem and Scholljegerdes, 1950—Series A; IV McLimans, Siem and Scholljegerdes, 1950—Series B; V McLimans, Siem, Mark and Pinska, 1950; VI Greiff and Pinkerton, 1948; VII Hasselbalch, 1900; VIII calculated from Murray, 1925, 1926.

† Standard deviation.

behavior of weighted blank divers were made. Even when runs were continued for periods longer than any of the experimental runs, no deviations of the recording pens were detected. Since the recorders in the control runs were set at sensitivities equal to the greatest ones used in the experiments, it could safely be assumed that no oxygen loss which was detectable by the methods used occurred during these experiments.

This study was concerned more with variations in rates of oxygen consumption than with absolute rates. However, comparative data concerning absolute measurements made with this apparatus have been compiled in Table I.

One problem in determining daily variations in oxygen consumption in chick embryos is the increasing overall rate of consumption. In order to examine hourly fluctuations on a comparative basis, a correction for this trend must be made. The method chosen for this study was the construction of a line of least squares fit by regression analysis (Simpson, Roe and Lewontin, 1960). Regression analysis was performed on every complete organism-calendar-day of data collected during the

course of the study. The resultant calculated values provided a basis for determining hourly deviation from trend. Each recorded hourly value was divided by the appropriate regression value to permit its description as a percentage of trend value. These percentage values made plotting of daily values on a non-sloping line possible and allowed more valid comparisons because all data were reduced to a common relationship. Therefore, despite differences in total oxygen consumption among individual organisms on a given day of incubation and between different days of incubation, meaningful examinations of similarities and differences in form of individual patterns and mean patterns of groups could be made. Unless otherwise stated, all results and discussion presented will relate to analysis of these regression relationships.

Several devices were employed in examining the daily patterns. Weighted (1, 2, 1) moving means (Croxtton and Cowden, 1955) were calculated from the hourly means in some instances. Means for consecutive three-hour periods were calculated in other cases. Ratios of the number of hourly recorded values falling above the trend line to the number falling below for given periods were also calculated.

RESULTS

The use of regression analysis and the calculation of per cent of trend values from data such as those collected in this study reduce all data to a common form and also make possible gross comparisons of relationships to the trend line. Since this analysis corrects for the continually increasing overall rate of oxygen consumption during ontogeny, it is possible to detect metabolic variations not accountable by simple ontogenetic increase. As the metabolism of the adult chicken has a strong diurnal variation, the per cent of trend values were first examined on the basis of an arbitrary separation of nighttime from daytime values (all 38° C., 9 PM incubation time data were used). In the 1 AM to 6 AM data 724 hourly values were found

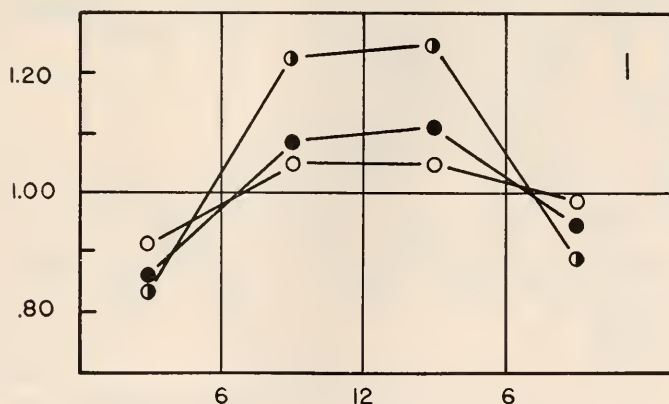


FIGURE 1. Ratios of the number of hourly values greater than 100% of trend values to the number of values less than 100% of trend calculated for six-hour periods. Closed circles represent all of the data gathered at 38° C. from embryos which were incubated initially at 9 PM (see text). Open circles represent the same data minus day-seven and day-eight data. Half-closed circles represent data gathered at 33° C.

to be above the trend line and 836 values were below it. In the 7 PM to midnight data 763 values were above and 797 values were below the trend line. The twelve daytime hours (7 AM-6 PM) showed a considerably different picture with 1624 values above 100% of trend and 1496 below it. Statistical comparison of the day and night values by use of Chi-square analysis indicates a significant difference ($\chi^2 = 12.04$, $P < 0.001$). Therefore, irrespective of any other complexities of form in the daily cycles, there appears to be a mean diurnal variation even at these early stages of development.

More refined examinations of these relationships can be accomplished in another way. A ratio of the number of hourly per cent of trend values falling above 100% of trend to those falling below that level provides a convenient numerical value for purposes of comparison. Figure 1 (closed circles) shows the ratios calculated using all hourly values for six-hour periods of the day.

The foregoing information is of value only in detecting gross relationships and it becomes necessary to use the actual per cent of trend values for more precise examination of characteristics of this metabolic variability. Ratios such as those seen in Figure 1 assign equal weight to all hourly values and do not take large percentage deviations into account. Separation of the data into three-hour periods using mean per cent of trend values yields some interesting differences among the days of incubation studied (Fig. 2). The patterns for days four, five and six show considerable similarities through the pre-dawn hours, but then a difference appears. Days four (2a) and five (2b) go on to afternoon highs and evening lows but a similar progression through the day is lacking in the day-six data (2c) except for

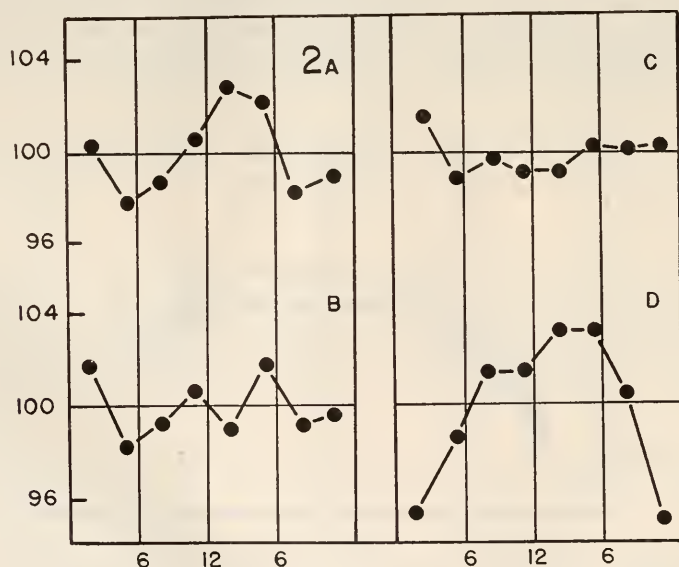


FIGURE 2. Means of hourly per cent of trend values calculated for three-hour periods of the day. The number of organism-calendar-days of data involved in each instance is indicated in parentheses. A. 38° day-four data (77). B. 38° day-five data (90). C. 38° day-six data (79). D. Combined 38° day-seven and day-eight data (12 day sevens and 7 day eights).

the merest suggestion. In the day-seven and day-eight data (2d) a new pattern is emergent. This seems to be a more emphatic expression of the gross pattern of day-night differences seen in the embryo throughout the period investigated. Since the data for days seven and eight are included in the ratios cited in Figure 1

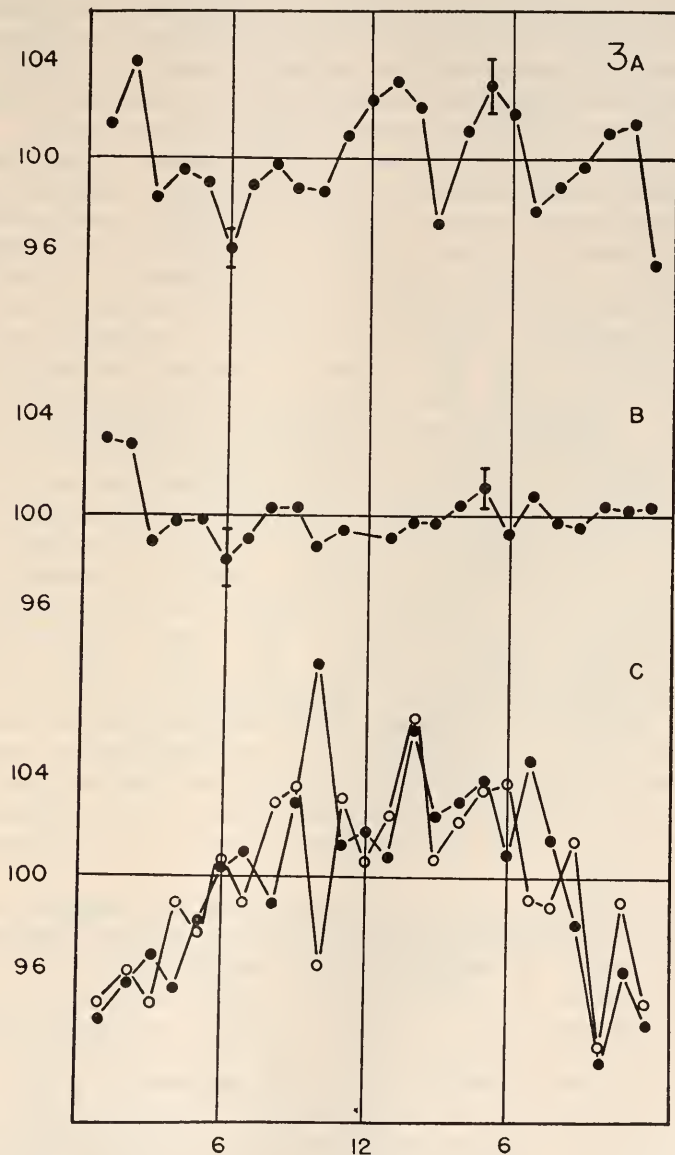


FIGURE 3. Hourly means of per cent of trend values. A. Day-four and day-five data from three seasons. B. Day-six data from three seasons. C. Day-seven (open circles) and day-eight (closed circles) data. Bars indicate standard errors of means.

(closed circles), the validity of this statement might be open to question. However, the same comparison can be made between the day-seven and day-eight pattern and that seen in the ratios which exclude day-seven and day-eight data (Fig. 1, open circles).

Figure 3 permits examination of these variations on an hour-by-hour basis. Because of the similarities seen in the patterns of variation in the day-four and day-five data, the values from both days are grouped together in Figure 3a. The apparently unique pattern of variation around the trend line seen in the day-six data is seen again in Figure 3b. The data from days seven and eight are plotted separately in Figure 3c to disclose their similarity to one another.

Another matter of concern in determining the nature of metabolic variability during development was the possibility that the daily cycle might have a different form at different times of the year. For all seasons the greatest number of uninterrupted respirometer-days was obtained for the fifth day of incubation. This day of incubation can serve as an index of seasonal fluctuation as the data should be fully comparable. Means of the per cent of trend values for three-hour periods of day five are presented in Figure 4 (a, b and c). Similarities are seen in the early morning and afternoon relationships, but a striking inversion in the morning values is seen with the spring means varying in a manner different from the fall and winter means. A comparable seasonal difference was evident for the fourth day of incubation. In order to show the daily cycle in day-five embryos in more detail, three-hour weighted (1, 2, 1) moving means of the hourly per cent of trend values are presented in Figure 5 (a, b and c). Differences among the mean daily cycles become obvious upon examination of this figure. The early morning hours (1 AM–4 AM values) in all three cases show a steady downward progression which begins well above the trend line. However, the similarity largely ceases here. In the spring, one of the highest values of the day occurs at 8 AM while a similar conspicuous increase is lacking in the fall and winter data. The pattern through the mid-part of the day in the spring is essentially inverted relative to that of the fall and winter data. In the fall and winter, a maximum occurs over the noon hour. A common characteristic of the daily cycles is a mid-afternoon low value which is seen in the data for all three seasons. This low value is followed in all cases by a sharp rise to a late afternoon high value occurring at 4, 5, or 6 PM depending upon the season of the year. The rate then drops off into an evening pattern which is similar in all seasons.

Experiments designed to reveal information about the temperature relationships of this metabolic variability were conducted in the fall. Control respirometers were run at 38° C. while other respirometers were maintained at 33° C. Eggs were transferred to both the control and the 33° C. respirometers on the third day of incubation. Data recorded on the fifth day will be emphasized here. At the beginning of day five the embryos in the 33° C. respirometers have already been subjected to 32 or more hours at the depressed temperature. Initial comparisons with control data from day five can be made by comparing Figures 6a and 5b. The daytime patterns of O_2 -consumption variation are quite similar (note 6 AM–8 PM values), but bear a different relationship to the 100% of trend line. This is due to the fact that at 33° C. the early morning and late evening values are markedly depressed and this depression is detected statistically in the form of changed rela-

tionships to the trend line. This early morning and late evening depression will be discussed more extensively later, but it should be borne in mind when focusing attention on the three-hour mean values (Fig. 4d). If the plots are compared point by point, it can be seen that the pattern of variation follows that of the control data (Fig. 4b) quite well, but the first predawn value and the two evening values are lowered in relation to the trend line. It should be noted especially that the direction of change from one value to the next is parallel in all cases except the one involving the first predawn value. Another aspect of the data taken at 33° C. to be noted is the similarity of the patterns of variation on day five and day six (Figs. 6a and b, 4e and d). Finally, the changed relationships to the trend line are obvious in the ratios of hourly values above 100% of trend to values below 100% which are presented in Figure 1 (half-closed circles). This is further indication of the relatively greater depression of the nighttime oxygen consumption by the lower temperature.

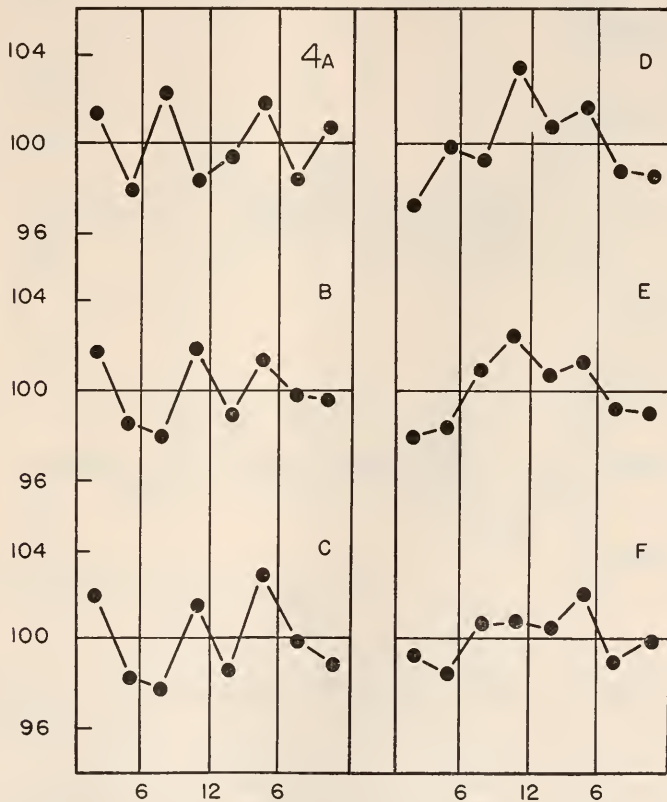


FIGURE 4. Means of hourly per cent of trend values calculated for three-hour periods of the day. The number of organism-calendar-days of data involved in each instance is indicated in parentheses. A. Spring 38° day five (30). B. Fall 38° day five (38). C. Winter 38° day five (22). D. Fall 33° day five (33). E. Fall 33° day six (35). F. Winter "9 AM" day five (16).

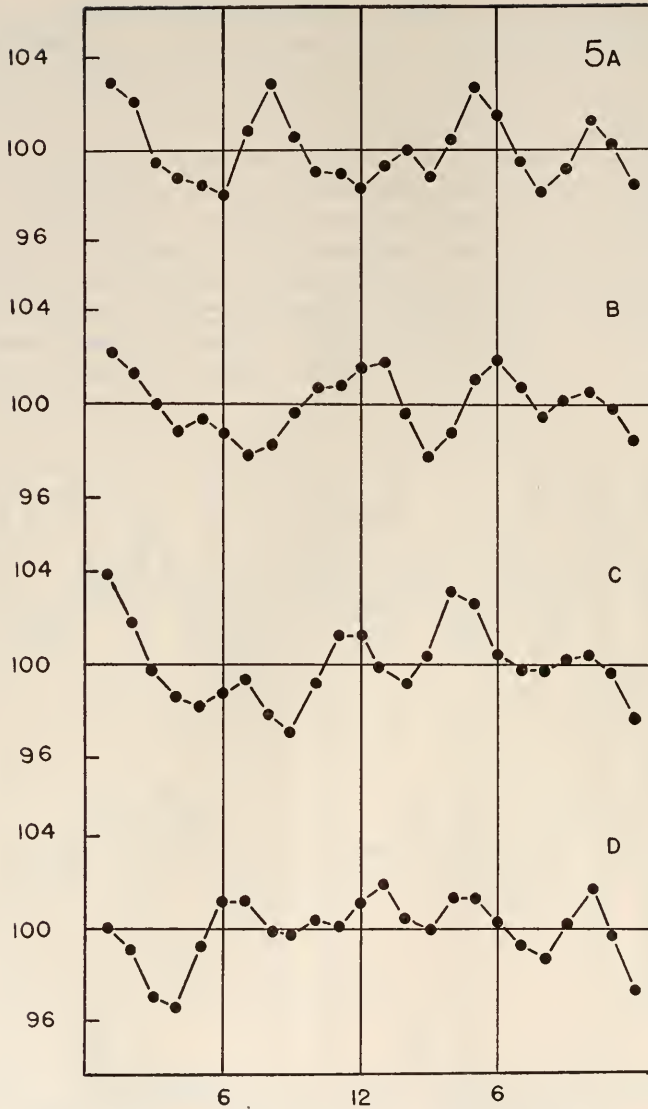


FIGURE 5. Three-hour weighted moving means (1, 2, 1) of hourly per cent of trend values. A. Spring 38° day five. B. Fall 38° day five. C. Winter 38° day five. D. Winter "9 AM" day five.

Data on the nature of metabolic variability when incubation was initiated at a different time of the day were obtained in winter. They are not as extensive as might be desired since only two times of incubation were considered. Embryos whose incubation was initiated at 9 AM were compared with embryos incubated, as in all previous experiments, at 9 PM. Only 16 complete organism-calendar-days of data were obtained for the "9 AM" group, but some suggestive conclusions are

possible. Again using day-five data for comparison it can be seen in the three-hour mean plots (Fig. 4f and c) that the pattern of variability is similar. For purposes of general comparison, attention is also called to the similarity between the "9 AM" day-five pattern and the overall mean day-five pattern (Fig. 2b). The weighted moving mean plots (Fig. 5d and c) show parallels in the early morning hours where the trend is downward. This is followed by a peak over 6 and 7 AM, a depression in mid-morning, and a peak over the noon hour. The remainder of the day's pattern can be followed in the same way. No explanation other than small

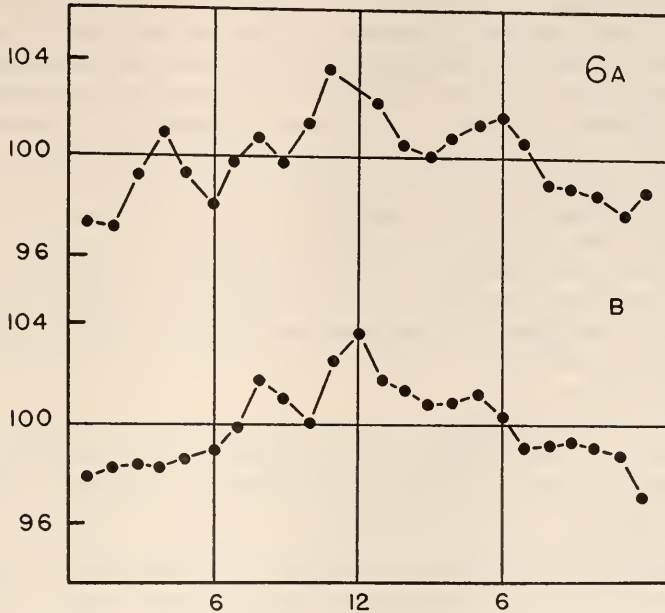


FIGURE 6. Three-hour weighted moving means (1, 2, 1) of hourly per cent of trend values.
A. 33° day five. B. 33° day six.

sample size is readily apparent for the altered relationships to the trend line, but the emphasis is placed on similarity of pattern.

DISCUSSION AND CONCLUSIONS

The data gathered in this study confirm the conclusion of Barnwell that systematic metabolic variations exist in the chick embryo as early as the first week of incubation. The present investigation of this phenomenon involved study of the forms of the daily patterns of variation from the fourth through the eighth days of incubation. The pattern seen during days four and five is one in which several peaks occur, but on days seven and eight there seems to be a somewhat simpler pattern while the pattern for day six is peculiar to that day. This could represent an evolution toward the form of the adult chicken metabolic rhythm which has been reported to be strongly diurnal (Bacq, 1929; Barott *et al.*, 1938). The nature of

the changes in the pattern of oxygen consumption seen through this period of development can only be described and any explanations of these changes must be deferred. However, it may be postulated that during this investigated period of development some overt, energy-requiring process becomes coupled into a genetically determined relationship with the underlying rhythmicity reflected in the early basal metabolic variability. If this coupling were a gradual process rather than an instantaneous one, it would provide one explanation of the failure of the day-six data to fit the pattern of either the days which precede or follow that day. Although it is possible that several or many processes might be involved in such a coupling, it has been reported that some important neuromuscular integrations are being established at about these stages of development (Hamburger, 1963; Hamburger and Balaban, 1963). Energy-requiring processes having a periodicity adopted from underlying rhythmicity might be related to this neuromuscular integration. Limb motility could be such a process. This basic rhythmicity is presumed to have been present from earliest stages of development and is available to the organism as a means of regulating various physiological processes. The organism appears to function to a certain extent as an amplifier and to produce large scale differences with periodisms adopted from this subtler underlying rhythmic system. The adult may function even more strongly as an amplifier system and this rhythmicity could be utilized to time numerous physiological events. By the time that the energy requirements of all of these processes have been met it would no longer be possible to use metabolic variability as an index of the true character of the basic rhythmic nature of the organism.

Results obtained during different seasons indicate that there is an annual component in the expression of the fundamental metabolic variability of the chick embryo. Study of a comparable stage of development (day five) at different times of the year demonstrates a seasonal variation in the form of the daily pattern of oxygen-consumption. It was noted that the daily patterns were very different from one another in spring and fall. Although the winter data were more similar to the fall data, in some ways they appeared suggestively to be intermediate between the spring and fall data. The pattern of annual variation seen in the three-hour means shows similarities to the pattern of annual variation reported for some other organisms. One of the most noticeable elements of seasonal differences is the marked change of pattern over midday. Such a seasonal noon difference was reported for the daily metabolic pattern in the potato. In that organism the noon values are relatively low points in the daily cycle during the spring and high points in the fall of the year (Brown, 1958, 1960). The inversion of the late morning mean (this mean includes the noon value) which occurs between the spring and fall data produces a similar picture in the chick embryo. The late afternoon maximum is very persistent in all seasons examined, just as reported for the potato. In organisms such as the potato (Brown, 1958) and bean (Lutsch, 1962) another aspect of seasonal differences is the variation in mean overall rate of oxygen consumption. Although very suggestive data indicating higher metabolic rates in the spring were obtained during this study, this aspect of metabolic variability in the chick embryo will require further work and such experiments have been planned.

Study of temperature relationships of the metabolic fluctuations produced some interesting results. Several lines of evidence indicate that the period length of the

basic metabolic rhythm is not altered by lowering the temperature of the organism. Although the temperature of the embryos had been lowered for 32 hours or more at the beginning of day five, fluctuations parallel those seen in the 38° C. organisms. This is particularly apparent in the pattern of variation seen in the daytime hours. Another interesting parallel is seen upon comparing the patterns of variability seen in the 33° C. day-five and day-six embryos (Fig. 6). These embryos on succeeding days show a remarkably similar pattern of variation. A question might be raised about the relationship of these data to those presented on ontogeny of the pattern of metabolic variability. It must be remembered in this connection that by the beginning of what is being called day six here the 33° C. embryos have been subjected to the lowered temperature for 56 hours or more and it is very difficult to say to what extent the previously discussed ontogenetic processes have been retarded by the lowered temperature. The conclusion drawn from these data is that the period of the metabolic rhythmicity seems to be temperature-independent or, at least, that the Q_{10} value for the period length is so close to 1.0 that any difference from that value is too small to be detected by the methods used in this study.

A second result of the lowered temperature is the suppression of the rate of oxygen consumption in the early and late portions of the 24-hour period (nighttime values) relative to the other values (daytime values). This suppression is detected through linear regression analysis as an altered relationship to the trend line. It is an interesting conjecture that even at these stages of development, there might be a regular periodic variation in the sensitivity of the organism's overall metabolism to temperature differences.

The similarity of the pattern of variation in organisms whose incubation was initiated at different times of the day demonstrates that there is no triggering associated with the beginning of incubation, but rather that the characteristic daily pattern will find expression even if the time of incubation is different. The patterns of variation shown by the "9 AM" chicks were not 12 hours out of phase with the "9 PM" chicks, but rather were in phase with them and with the time of day even under laboratory constant conditions. The differences observed between the small sample of "9 AM" chicks (16 organism-calendar-days) and the controls are probably due to individual variation or to the fact that ontogenetic factors previously discussed may obscure the results since the "9 AM" chicks are 12 hours younger in incubation age than the "9 PM" chicks. In this connection, attention is called to the overall pattern of variation in day-five embryos (Fig. 2b) and also to the day-four pattern (Fig. 2a).

The properties of this metabolic variability are of interest in themselves because of their implications concerning the ontogeny of periodicity, but they also have some bearing on more general aspects of the biological clock problem. A long-standing controversy in this field has been the support by various workers in the field of two different hypotheses concerning the nature of the timing mechanism of biological rhythms. The hypothesis of exogenous timing related to geophysical environmental factors has been supported by Brown and others (Brown, 1959b, 1960, 1962) in recent years. An alternative hypothesis is that expressed by Pittendrigh and others (*e.g.*, Pittendrigh and Bruce, 1957, 1959; Aschoff, 1963) which views the timer mechanism of biological clocks as an autonomous, endogenous oscillator

system which is physico-chemical in nature. The merits of, and evidences for, these viewpoints have been discussed extensively in a recent work by Brown (1965).

The possession of seasonal differences in the daily patterns of variability has some bearing on this question. Chick embryos whose development is initiated at one time of the year and others initiated at other times of the year show decidedly different patterns of daily variation in oxygen consumption and these seasonal patterns parallel those seen in other organisms. In the case of an autonomous internal timer, the genetic mechanisms which must be hypothesized to account for such seasonal differences would have to be fantastically complex. It seems simpler and more direct to hypothesize a basic responding system which when it is examined, even in embryonic stages, shows seasonal patterns of response reflecting seasonal differences in variations in geophysical factors.

The significance of the relative temperature-independence of the period-length of biological rhythms has been discussed extensively in the past. Proponents of the autonomous internal oscillator hypothesis assume that such an inherent oscillator system would be capable of temperature compensation. Problems are posed for this viewpoint because this type of temperature-independence is not ordinarily seen in biological chemical reactions where Q_{10} values of 2.0–3.0 and even higher are common. An alternative explanation which is consistent with the observed facts could be that a basic responding system exists and that external time cues continue to be available to the organism with regularity, no matter what the temperature of its immediate environment.

The apparent lack of a triggering point demonstrated by the experiments where incubation was initiated at different times of the day provides another piece of evidence related to the nature of the biological clocks. One group of embryos was essentially 12 hours out of phase with the other group in their relationship to the actual time of day at the beginning of incubation. The strikingly similar patterns of metabolic variations seen in the two groups is again suggestive of an open receptor system which responds to an input related to the exact time of day even under laboratory constant conditions.

I am grateful to Professor Frank A. Brown, Jr. for his advice and constructive criticism during the course of this study. Work in Professor Brown's laboratory was supported by grants from the National Science Foundation (GB-469) and the National Institutes of Health (GM 07405) and by a contract with the Office of Naval Research (1228-30). I was supported by a National Science Foundation Predoctoral Fellowship.

SUMMARY

1. Continuous recordings of oxygen consumption were made in order to determine ontogenetic, daily, seasonal, temperature, and other relationships of metabolic variability in chick embryos.

2. Statistical treatment involving linear regression analysis facilitated resolution of daily cycle forms and allowed various comparisons.

3. The pattern of metabolic variation during days four and five has several peaks, but by days seven and eight a more markedly diurnal pattern appears. Day six appears to be an intermediate or transitional stage.

4. Seasonal differences in the form of the daily cycle resemble those reported for other organisms.
5. Lowering the temperature 5° C. does not affect the period length of the metabolic variations. A suppression of nighttime metabolism relative to daytime metabolism suggests the expression of a diurnal variation in temperature sensitivity.
6. Initiation of incubation at different times of day does not result in different basic cycle forms.
7. Results obtained with chick embryos are suggestive of a receptor system which is responsive to external time cues.

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THE ROLE OF DNA SYNTHESIS IN THE DETERMINATION OF AXIAL POLARITY OF REGENERATING PLANARIANS¹

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It has been shown that treatment of intact planarians with colcemide (deacetyl-methylcolchicine) and chloramphenicol, followed by cutting the worms to remove the heads and tails, will cause the regeneration of bipolar heads in a significant number of these cut planarians (Flickinger, 1959). When applied locally to the prospective anterior ends of cut pieces of worms, these compounds could reverse the normal polarity of the worms (Flickinger, 1959; Flickinger and Coward, 1962). It was further shown that both of these compounds inhibit the extent of incorporation of $C^{14}O_2$ into a trichloroacetic acid-insoluble residue, which is primarily protein. The classical action of colchicine, or its derivatives, is to inhibit cell division, while the action of chloramphenicol in bacteria is to inhibit protein synthesis without significantly inhibiting nucleic acid synthesis (Gale and Folks, 1953; Midgley and McCarthy, 1962). An accumulation of RNA in bacteria treated with chloramphenicol has been observed (Dubin and Elkart, 1965) and such RNA has some similarities to messenger RNA (Hahn and Wolfe, 1962).

One purpose of this investigation was to find the effects of chloramphenicol upon RNA and DNA synthesis of the planarians. Does chloramphenicol stimulate or inhibit RNA synthesis? Is there an inhibition of DNA synthesis by chloramphenicol? If so, what is the per cent of inhibition of DNA synthesis after periods of exposure which cause bipolar head formation, compared to shorter times of exposure which do not affect polarity of the regenerating worms? Another aim of the present investigation was to determine the temporal course of DNA, RNA and protein synthesis in the regenerating head and tail blastemata during the first 24 hours after cutting the worms. A study of RNA and protein synthesis in regenerating planarians has been made (Coward and Flickinger, 1965), but these determinations were made at daily intervals for a 7-day period.

MATERIALS AND METHODS

The planarians, *Dugesia tigrina*, maintained in aerated tap water, were starved for 7–10 days before being used in an experiment. The effect of chloramphenicol upon RNA and DNA synthesis was tested by exposing 100 worms for 12 or 24 hours to 25 μ c./ml. of uridine-2- C^{14} or C^{14} -thymidine in 1 ml. of boiled tap water containing chloramphenicol succinate (1.5 mg./ml.). A similar number of control worms was cultured for similar periods in boiled tap water, containing the labeled compounds. At the conclusion of the incubation period the worms were washed five times with boiled tap water, by which time the final rinse water had a level

¹ This investigation was supported by a grant from the National Science Foundation.

of activity similar to the background. The worms were then homogenized in cold 5% trichloroacetic acid (TCA) and the pellet washed three times by centrifugation, which reduced the level of isotopic activity of the wash to that of the background. The residue was extracted twice with 1:1 ethanol-ether, twice with 1:4 ethanol-ether, and once with ether alone to remove lipids. The residues were then dried. For the C^{14} -uridine experiments RNA was hydrolyzed with 1 ml. of 0.3 N KOH at 37° C. for 20 hours. The hydrolysate was acidified with 0.3 ml. of 1 N perchloric acid to precipitate the DNA and protein and the supernatant was neutralized with 1 N KOH to precipitate the perchlorate. The concentrations of RNA were determined by the orcinol method (Dische, 1955) and the samples were plated and counted on a thin window proportional counter. The procedure for the C^{14} -thymidine incubations was similar except that both RNA and DNA were hydrolyzed with hot trichloroacetic acid (96° C.) for 40 minutes and the hydrolysate was extracted three times with ether to remove the trichloroacetic acid. The diphenylamine method (Dische, 1955) was used to determine DNA concentration before the samples were plated and counted.

For the incubations with $C^{14}O_2$ 100 cut worms were transferred to 1 ml. of recently boiled tap water in a small dish. A small amount of lactic acid was added to generate $C^{14}O_2$ from 150 μ c. of $BaC^{14}O_3$ in a center well and the dish was quickly sealed to prevent the escape of $C^{14}O_2$. After the incubation, the worms were processed as before for hydrolysis of RNA with 0.3 N KOH, and DNA of the residue was hydrolyzed with hot TCA. The protein residue from the DNA hydrolysis was dissolved in formic acid, the protein concentration determined (Lowry *et al.*, 1951) and then this fraction was plated and counted.

RESULTS

Biological experiments with chloramphenicol

In experiments designed to find the minimal concentration of chloramphenicol that would produce bipolar heads in regenerating planaria, 50 whole worms were incubated in boiled tap water containing 0.1% penicillin and streptomycin and 50 were placed in a similar solution containing chloramphenicol succinate (1.5 mg./ml.). This concentration of chloramphenicol succinate is equivalent to 1 mg./ml. chloramphenicol. Similar numbers of worms were cultured in 0.75, 0.325 and 0.167 mg./ml. of chloramphenicol succinate. After 24 hours of incubation the worms were each cut just back of the head and in front of the pharynx and the cut pieces were allowed to regenerate in boiled tap water containing 0.1% penicillin and streptomycin. Thirty per cent of the cut pieces in chloramphenicol succinate (1.5 mg./ml.) subsequently developed heads at each cut end, but no bipolar heads were observed in the pieces allowed to develop in the boiled tap water alone or in the lower concentrations of chloramphenicol. In order to learn if the polarity of the regenerating worms could be affected by exposure to chloramphenicol after the worms were cut, 60 worms were cut in a similar fashion to provide pre-pharyngeal pieces and these were then placed in chloramphenicol succinate (1.5 mg./ml.) for 24 hours. Subsequent culture of these regenerating pieces provided only two cases of bipolar head formation.

To determine the minimal period of exposure to chloramphenicol necessary to

produce bipolar heads, groups of 20 whole worms were incubated in chloramphenicol succinate (1.5 mg./ml.) for periods of 2, 8, 12, 16, 20 and 24 hours and the pre-pharyngeal pieces were obtained by cutting the worms. These were allowed to regenerate in boiled tap water containing 0.1% penicillin and streptomycin and the water was changed each day. No cases of bipolar heads occurred in worms exposed to chloramphenicol succinate (1.5 mg./ml.) for less than 20 hours. Two of the worms exposed for 20 hours and eight of those exposed for 24 hours regenerated heads at each end.

Isotopic experiments with chloramphenicol

Previous work had shown that chloramphenicol inhibits the incorporation of $C^{14}O_2$ into the trichloroacetic acid-insoluble fraction of planarians (Flickinger, 1959). To ascertain the effect of chloramphenicol upon RNA synthesis, 100 intact worms were incubated separately in 1 ml. of boiled tap water containing chloramphenicol succinate (1.5 mg./ml.) and 25 μ c./ml. of uridine-2- C^{14} for 24 hours at 18° C. One hundred control worms were incubated similarly except that chloramphenicol was not present. The RNA was hydrolyzed and counted and the results show that worms in chloramphenicol incorporated less C^{14} -uridine into the RNA fraction (Table I). There was a 24.8% inhibition based on the activity/100 worms, while there was a 28.8% inhibition on the basis of specific activity.

A similar type of experiment was performed to find the effect of chloramphenicol upon DNA synthesis. One group of 100 worms was incubated in boiled tap water containing 25 μ c./ml. of C^{14} -thymidine and chloramphenicol succinate (1.5 mg./ml.) for 24 hours at 18° C. while the incubation of the control group of 100 worms did not contain chloramphenicol. Another two groups of 100 worms were incubated separately for 12 hours with 25 μ c./ml. of C^{14} -thymidine, and chloramphenicol succinate (1.5 mg./ml.) was present in one of those incubations. From the biological experiments it was known that a 24-hour exposure to this concentration of chloramphenicol would produce bipolar heads in regenerating worms, while the 12-hour exposure had no effect upon polarity. This experiment offered the chance to compare the effect of the biologically active and inactive doses of chloramphenicol upon the incorporation of C^{14} -thymidine into DNA. The results of this experiment are given in Table II. There was a 39.1% inhibition of C^{14} -thymidine incorporation into DNA with a 24-hour exposure to chloramphenicol, and a 10.3% inhibition following a 12-hour exposure.

TABLE I

Effect of chloramphenicol upon incorporation of C^{14} -uridine into RNA of intact planarians. Incubation of 100 worms for 24 hours at 18° C, in boiled tap water containing 25 μ c./ml. of uridine-2- C^{14} , and similar incubation of another 100 worms with chloramphenicol succinate (1.5 mg./ml.) present

	$\frac{\text{Cpm}}{100 \text{ worms}}$	$\frac{\text{Cpm}}{\text{mg. RNA}}$	Per cent inhibition of specific activity
Control worms	4378	8420	
Chloramphenicol succinate (1.5 mg./ml.) worms	3303	6000	28.8

TABLE II

Effect of chloramphenicol upon incorporation of C^{14} -thymidine into DNA of intact planarians. Incubation of four groups of 100 worms for 12 and 24 hours at 18° C. in boiled tap water containing 25 μ c./ml. of C^{14} -thymidine. Chloramphenicol succinate (1.5 mg./ml.) was present in one of the 12-hour and one of the 24-hour incubations

	Cpm 100 worms	Cpm mg. RNA	Per cent inhibition of specific activity
Control, 12 hours	447	1241	
Chloramphenicol succinate, 12 hours	368	1115	10.3
Control, 24 hours	598	1245	
Chloramphenicol succinate, 24 hours	303	758	39.1

DNA, RNA and protein synthesis of blastemata

Exposure of cut worms to chloramphenicol for 24 hours did not alter their regeneration polarity, while a similar period of exposure of whole worms to chloramphenicol, followed by cutting to obtain pre-pharyngeal pieces, produced bipolar heads in 40% of the regenerating worms. This suggested that the mechanisms accounting for establishment of normal polarity operate during the first 24 hours after cutting the worms. The rates of DNA, RNA and protein synthesis during this period were examined in the following manner. For each experiment 100 worms were cut transversely in front of the pharynx at level X (Fig. 1) and the anterior and posterior parts of these worms were allowed to regenerate for 2, 4, 6 or 24 hours in boiled tap water. $C^{14}O_2$ was generated from 150 μ c. of $BaC^{14}O_3$ and the dish was sealed and incubated at 18° C. for three hours. The cut worms were then washed five times with boiled tap water, fixed in 5% TCA and cut into A_2 , A_1 , P_1 , and P_2 parts, according to Figure 1. The head and tail blastemata (P_1 and A_1), and the areas of tissues adjacent to the head and tail blastemata (P_2 and A_2) were homogenized separately in cold 5% TCA and the centrifugal residues were washed four times by centrifugation and washing with cold 5% TCA. The DNA, RNA and protein fractions were prepared according to previously outlined methods and these fractions were plated and counted. Orcinol tests revealed the absence of RNA in the DNA fraction and the diphenylamine reaction showed there was no DNA in the RNA fraction. Examination of the results (Table III) reveals only slight differences of incorporation of $C^{14}O_2$ into DNA, RNA and protein of the four areas of the worms which had regenerated two hours without label and three hours with the label. The blastemata (P_1 and A_1) and adjacent areas (P_2 and A_2) obtained from cut worms that had regenerated 4 and 6 hours without label, plus three hours in labeled $C^{14}O_2$, showed significant differences in the levels of incorporation of label into DNA, RNA and protein. By this time the DNA, RNA and protein fractions of regenerating areas (P_1 and A_1) had higher isotopic activities than the non-regenerating areas (P_2 and A_2). After 6 hours of regeneration without $C^{14}O_2$, and three hours with $C^{14}O_2$, the head blastema (P_1) fractions (DNA, RNA and protein) incorporated more label than the tail

blastema (A_1) fractions. The non-regenerating areas near the head blastemata had higher activities than similar areas near the tail blastemata ($P_2 > A_2$). The greatest amount of $C^{14}O_2$ incorporation into DNA occurred at 4 hours for the tail blastemata (A_1) and the adjacent area (A_2), while the incorporation of label into both DNA and RNA of all the other fractions was greatest at 6 hours. In comparing the levels of isotope incorporation of the worms allowed to regenerate two hours in unlabeled medium and three hours in $C^{14}O_2$ with those in which the worms regenerated four or six hours in unlabeled medium and three hours in $C^{14}O_2$, significant increases of incorporation of labeled precursor into DNA and RNA were observed in the four- and six-hour experiments. Furthermore, there is a marked stimulation of DNA synthesis in the blastemata of worms that regenerated four hours in unlabeled medium, but no further significant stimulation of labeled precursor into DNA after six hours of regeneration in unlabeled medium. The extent of stimulation of RNA synthesis, particularly in the anterior pieces (P_1 and P_2), was greater between four and six hours of regeneration in unlabeled medium, as compared to the difference between two and four hours of regeneration in the unlabeled medium. The specific activities of the protein fraction were maximal after 24 hours of regeneration in tap water and three hours with the label. The differences in isotopic activities of the protein fraction between the head and tail blastemata

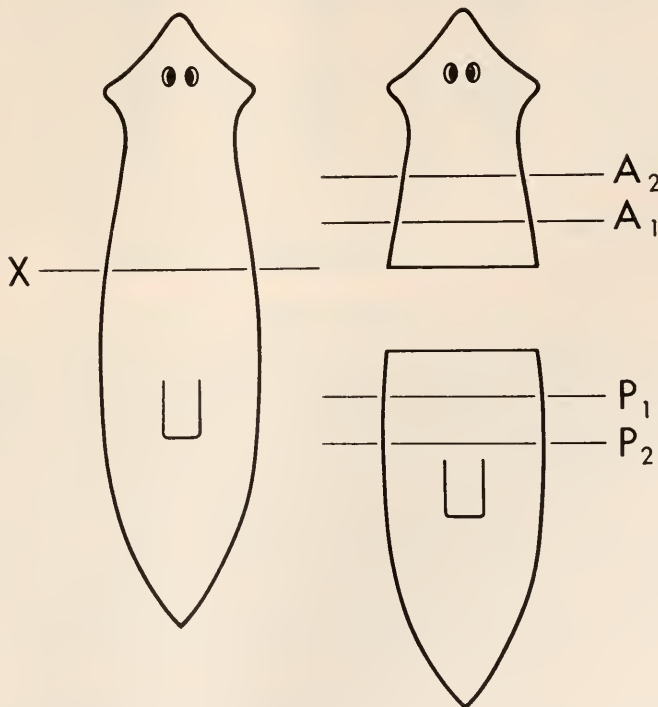


FIGURE 1. For each experiment 100 worms were cut at level x and allowed to regenerate for 2, 4, 6 and 24 hours. The blastemata (A_1 and P_1) and adjacent areas (A_2 and P_2) were cut and then incubated separately for three hours in 1 ml. of boiled tap water containing $C^{14}O_2$ generated from 150 μ c. of $BaC^{14}O_3$.

TABLE III

Incorporation of $C^{14}O_2$ into DNA, RNA and protein of head and tail blastemata and adjacent regions during the first 24 hours of regeneration. For each experiment 100 worms were cut anterior to the pharynx (Fig. 1) and the pieces allowed to regenerate for 2, 4, 6 or 24 hours at 18°C.

The pieces (A_1 , A_2 , P_1 , P_2) were then cut according to Fig. 1 and the pieces of each kind were incubated for 3 hours at 18°C. in 1 ml. of boiled tap water with $C^{14}O_2$ generated from 150 μ c. of $BaC^{14}O_3$

Tissue		Cpm mg.			
		Time			
		2 hrs.	4 hrs.	6 hrs.	24 hrs.
DNA					
Tail blastema	A ₁	14370	35140	33900	18700
	A ₂	15140	27290	25600	16800
Head blastema	P ₁	14340	39780	40800	22760
	P ₂	13500	25680	29400	13660
RNA					
Tail blastema	A ₁	17860	25350	32430	30913
	A ₂	15230	21300	26955	22220
Head blastema	P ₁	18490	21390	43250	35880
	P ₂	17640	17160	28980	28500
Protein					
Tail blastema	A ₁	1370	1520	1285	1820
	A ₂	1250	1088	1080	1340
Head blastema	P ₁	1445	1863	1760	2840
	P ₂	1130	1187	1440	2600

($P_1 > A_1$), the adjacent areas ($P_2 > A_2$), and the regenerating and non-regenerating areas ($P_1 > P_2$; $A_1 > A_2$), were also maximal at 24 hours.

DISCUSSION

The results of this investigation show that dosages of chloramphenicol succinate (1.5 mg./ml. for 24 hours) that can induce the formation of bipolar heads in regenerating planarians inhibit RNA and DNA synthesis (Tables I and II). The inhibition of incorporation of C^{14} -uridine into RNA (Table I) argues against the idea that the morphogenetic activity of chloramphenicol is due to an accumulation of RNA, as can occur in bacteria (Dubin and Elkart, 1965). Incubation of the worms with labeled thymidine and chloramphenicol succinate (1.5 mg./ml.) for 24 hours revealed a severe inhibition (39.1%) of DNA synthesis (Table II). Exposure of whole worms to this same concentration of chloramphenicol for 12 hours does not alter the normal polarity during subsequent regeneration and this length of exposure to chloramphenicol resulted in a 10.3% inhibition of DNA synthesis (Table II). The data from these experiments show that a marked inhibition of DNA synthesis by chloramphenicol is essential in order to alter the polarity of the regenerating worms. Previous work has shown that exposure of intact worms to the mitotic poison, colcemide, can affect the polarity of these worms during their

regeneration (Kanatani, 1958; Flickinger, 1959) and emphasizes that cell division is a critical factor for maintenance of normal polarity.

Exposure of cut worms to chloramphenicol succinate (1.5 mg./ml.) for 24 hours did not alter their normal polarity. This agrees with the results of Kanatani (1958) who found that colcemide did not alter polarity when applied to cut worms. This suggests that the critical events that maintain polarity in cut pieces of planarians are established sometime during the first 24 hours after the worms are sectioned. Investigation of $C^{14}O_2$ incorporation of head and tail blastemata (P_1 and A_1), and adjoining areas (P_2 and A_2), revealed that DNA, RNA and protein synthesis are stimulated 7–9 hours after cutting the worms. Furthermore, the head blastema (P_1) has a significantly higher incorporation of $C^{14}O_2$ into these fractions than the tail blastema (A_1) by this time. In reference to the 2–5-hour levels of incorporation the greatest stimulation of DNA synthesis occurred 4–7 hours after cutting and the maximal increase of RNA synthesis from 6–9 hours after cutting, while the maximal incorporation of $C^{14}O_2$ into the protein fractions occurred 24–27 hours after cutting. It appears that the rate of protein synthesis is maximal at the time of actual differentiation while the rates of DNA and RNA synthesis increase during the period of the determination of axial polarity. It does seem that the time of greater stimulation of DNA synthesis precedes the time of maximal stimulation of RNA synthesis, using the two-hour period of regeneration in unlabeled medium as the reference.

It has been found that the action of chloramphenicol in producing bipolar heads is correlated with a severe inhibition of DNA synthesis, and that one of the first metabolic activities to show maximal stimulation during regeneration is DNA synthesis. This suggests that cell division plays an important role in the establishment of axial polarity in planarians. However, cytological data relating to mitotic frequency are necessary to confirm this suggestion.

SUMMARY

1. Levels of chloramphenicol which can produce bipolar heads in regenerating planarians were found to inhibit severely the synthesis of both DNA and RNA. A shorter period of exposure to chloramphenicol, which did not affect the polarity of the regenerating worms, produced only a slight inhibition of DNA synthesis.

2. Incubation of blastemata and adjacent areas of regenerating planarians with labeled CO_2 for periods of 2, 4, 6 and 24 hours revealed a maximal stimulation of DNA at 4–7 hours and of RNA at 6–9 hours after the worms were cut. It is at this time that the blastemata destined to form heads attain a higher level of DNA and RNA synthesis than the blastemata which will form tails. The maximal stimulation of protein synthesis occurred 24–27 hours after cutting the worms.

3. The necessity of obtaining a severe inhibition of DNA synthesis with chloramphenicol in order to produce bipolar heads in regenerating worms, as well as the sequential nature of DNA, RNA and protein synthesis in the regeneration blastemata, suggests that the stimulation of DNA synthesis is involved in the establishment of polarity of regenerating worms. These patterns of DNA synthesis may reflect the incidence of cell division during this period.

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ANALYSIS OF SOME TEMPERATURE EFFECTS ON DROSOPHILA PUPAE

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Day-old *Drosophila* pupae respond to high temperature treatment in a variety of ways. Three classes of response have been studied (Milkman, 1962, 1963). Adaptive responses follow relatively gentle treatments; morphogenetic changes result from moderate treatments; and more severe treatments lead to death. Even when observations are restricted to genetically uniform *D. melanogaster* pupae, raised under standard conditions at 23° C. for 25 hours after puparium formation, the qualitative diversity of response is impressive (Milkman, 1962). Considering the wings alone, reduction in size, anterior crossvein defects, posterior crossvein defects, holes, approximation of the third and fourth longitudinal veins, and adventitious appearance of vein material are each stereotyped responses to certain treatments; and these effects do not appear in order, depending simply upon duration of treatment; rather, there are specific and clear-cut qualitative associations between treatment temperature and response. Death, too, appears in unexpected ways; for example, 4¼–4½ hours at 37.5° is lethal to males. The same durations at 38.0° are not. Moreover, the kinetics of the responses are often unusual: the morphological response to 36.5° reaches a peak at 200 minutes, then drops.

This welter of phenomena is forbidding indeed, but there is a body of responses to a large category of treatments that is accessible to analysis. These responses have already been ordered tentatively; the present paper is largely to confirm the order, to add substance and detail, and to lay the foundation for a quantitative scheme capable of defining the kinetics of the several underlying events for a wide range of simple and complex temperature treatments. The purpose of such a scheme is two-fold: to serve as a clear model of complex temperature responses and to summarize the great number of observations that have been made in the present study.

We shall be concerned with the production of posterior crossvein defects at temperatures above 39.0°, with death, and with the protection against these responses conferred by exposure for certain durations to temperatures above 27°.

To review the evidence and conclusions presented to date, we can consider the results of treatments of various duration at 40.5°. It will be useful to refer to Figure 1. Very short treatments (5–10 seconds) followed by an interval at 23° increase resistance to killing and to the production of crossvein defects by subsequent exposures to high temperatures. This resistance is *transient*, wearing off in a couple of hours. We speak of two transitions, A → B at 40.5° and B → C (transient protection) at 23°. By comparing the durations at temperatures between 28° and 40.5° which produce effects similar to those of 5 or 10 seconds at

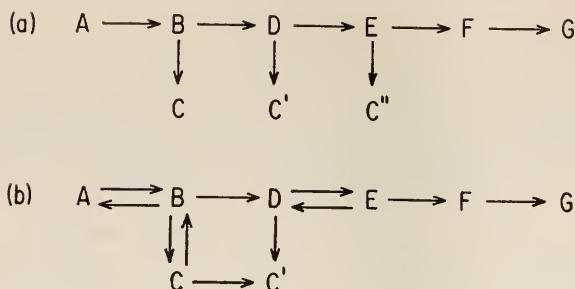


FIGURE 1. The total scheme as originally presented (A) and in its present form (B). The arrows represent transitions considered possible between the hypothetical states represented by letters.

40.5°, we assign $A \rightarrow B$ a one-degree temperature coefficient (Q_1) of 1.5. $B \rightarrow C$ is independent of temperature in the range observed.

Similarly, durations of 20–120 seconds at 40.5° followed by an interval at room temperature produce a *lasting* increase in resistance. The lasting nature of the protection is quantitatively inconsistent with the simple notion that the $A \rightarrow B \rightarrow C$ process has proceeded to a greater extent; therefore $B \rightarrow D$ at 40.5° and $D \rightarrow C'$ (lasting protection) at room temperature are postulated. This time a Q_1 of about 1.8 is obtained for $B \rightarrow D$. $D \rightarrow C'$ is again temperature-independent ($Q_1 = 1.0$).

A longer initial duration at 40.5° (on the order of 5 minutes) enables a subsequent treatment at temperatures down to 32° to produce posterior crossvein defects. This is related to a $D \rightarrow E$ transition. An interval at 23° still increases resistance to subsequent treatment. An $E \rightarrow C''$ step originally suggested has an alternative for which there is now evidence: a reverse change, $E \rightarrow D$, followed by $D \rightarrow C'$.

After a longer period (20 minutes) at 40.5°, an interval at 23° no longer confers protection, and so we have $E \rightarrow F$. But the appearance of defects begins only some time after this transition is essentially complete, so $F \rightarrow G$. Thus it is only in the transition $F \rightarrow G$, in the present scheme, that heat treatments result in crossvein defects. The total scheme as originally presented (Milkman, 1963) is shown in Figure 1a. In the light of additional evidence, a modified form seems more probable (Fig. 1b). The individual transitions here are not thought of as between successive, discrete physiological states, but rather as overlapping conversion processes at a level underlying the physiological states. As a working hypothesis, supported but unprovable by kinetics alone, it is supposed that the transitions are among several tertiary states of a single protein. The letters in the scheme can be taken to stand for these tertiary states, and it is possible to think of several existing at one particular time. In any pupa, the number of molecules in each tertiary state would determine the observable physiological state.

Naturally, such a set of conversions will under many circumstances of time and temperature present a complex picture (see Figure 2 in Hille & Milkman, 1966). The careers of various states will be hard to extricate. So in designing the experiments to be described, the general purpose has been to seek out the circumstances when one particular process stands out, and to study it; for with diverse temperature coefficients the various processes relate differently to one another at various tem-

peratures—being prominent in some and obscure in others. How the appropriate conditions were found will be described for each experiment.

In this paper, it will be impossible to ignore certain assumptions relating the transitions: that they connect the branched sequence of formal compartments (A, B, C, etc.); that they have first order kinetics; and that temperature affects only the rate of individual processes. These assumptions will, of course, have some significance in what is said. But the direct description and test of the set of relationships proposed is made in another paper (Hille and Milkman, 1966). For now, explicit discussion will be limited essentially to the individual steps and their kinetics.

MATERIALS AND METHODS

An inbred Oregon R strain of *Drosophila melanogaster* was used for all the experiments. Flies were raised in an incubator at 23°–25° until puparium formation, then in a Precision water bath at 23° until treatment. After treatment the pupae were returned to the 23° water bath for at least 12 hours and allowed to complete their development in the incubator at 23°–25°. The temperature was thus very closely controlled during the period when it is known to influence posterior crossvein formation and controlled fairly well throughout the life-cycle.

Water baths were controlled with Micro-set thermoregulators and monitored from time to time with thermometers calibrated to 0.01°. A YSI telethermometer was used with a thermistor (time constant 0.8 sec.) to confirm the thermal uniformity of the various regions of the bath. Warmup time (room temperature to within a degree of 40.5°) for pupae in teabags was shown to be less than 4 seconds. Even this technique was not sensitive enough, so more recently, unambiguous histochemical studies of the thermal inactivation of succinic dehydrogenase were used to show that larvae in teabags reach within 1° of 64° in less than 2 seconds.

Treatments totalling less than 40 minutes were generally made in teabags; longer ones employed vials, or else pupae were treated first in teabags and then transferred to vials for completion of the treatment. Crossvein defects were rated from 0 (normal) to 12 (both posterior crossveins completely absent) as previously described (Milkman, 1963).

RESULTS

Early protection

We shall speak of C and C' as protected states because they are off the effective pathway from A to the damaging state G. The distinction between C and C', and therefore between B and D, rests on the transience of protection due to C, as opposed to the lasting nature of protection due to C'. It has been shown that very short exposures to 40.5° at times between 21 and 24 hours, followed by an interval at 23°, protect against crossvein defect production by subsequent exposure to 40.5°. The effect of interval length is distinguishable from that of the pupal age at which the protective treatment was given (Milkman, 1963). As C protection wears off, the comparison in terms of protection conferred between short-interval and long-interval treatments permits the resolution of C formation from C' formation. The lasting protection found after longer intervals requires a longer initial treatment at 40.5°. In order to reconcile a long interval with a test treatment at the time

of peak sensitivity, 25 hours, the pretreatments were originally given at 21 hours (Milkman, 1963). More recently, in anticipation of making a unifying model for the events at 25 hours, these experiments were repeated in detail with pretreatments at 24 hours and test treatments at $25\frac{1}{2}$ hours. The results were essentially identical. Therefore, although the kinetics of at least one later step in the overall sequence must be different at the two ages (see the age-response curve for defect production [Milkman, 1962]), the kinetics through D formation are similar.

TABLE I

Onset and decline of quick protectability at 40.5° ; y seconds at 40.5° + x minutes at 23° + 18 minutes at 41.5° data expressed in rating units

y		x								
		$\frac{1}{2}$	1	2	3	4	5	6	8	10
0	M	5.7	5.8	5.4	5.6	4.3	3.8	5.1	3.4	1.8
	F	6.5	6.7	6.9	6.5	6.2	6.6	6.2	7.2	3.2
5	M	5.4	5.5	4.8	4.5	4.1	1.7	2.0	0.3	0.0
	F	6.1	6.4	6.1	5.9	6.7	5.0	3.0	1.4	0.3
10	M	4.5	5.1	4.1	3.9	2.4	2.1	1.0	0.1	0.1
	F	5.2	6.3	6.4	5.8	5.2	4.6	1.9	0.9	0.3
15	M	4.8	4.5	4.6	2.1	2.5	2.1	0.5	0.1	0.3
	F	5.8	5.9	5.9	4.3	4.4	2.9	2.0	0.6	0.7
20	M	4.9	5.1	5.0	4.1	2.4	1.6	0.4	0.1	0.0
	F	6.1	5.6	6.1	5.3	4.9	3.6	1.5	0.2	0.6
30	M	4.8	5.7	4.5	4.7	2.7	1.5	0.3	0.2	0.1
	F	5.3	5.1	4.7	4.9	4.4	3.5	1.8	0.4	0.1
60	M	4.6	3.8	2.9	4.6	1.9	1.2	0.1	0.3	0.1
	F	5.1	4.7	4.5	4.9	3.9	3.0	2.5	1.4	0.1
120	M	3.6	5.4	3.5	4.2	2.7	2.3	1.2	0.6	0.2
	F	4.4	5.5	4.2	4.6	3.5	4.1	3.1	1.7	0.8
180	M	5.0	5.6	5.6	5.2	5.4	3.3	2.0	0.8	0.3
	F	4.7	4.9	6.9	6.2	6.4	3.9	3.9	3.2	1.4
240	M	5.8	6.4	6.5	6.2	5.5	5.5	4.4	3.4	1.6
	F	7.0	7.1	7.8	7.9	7.0	6.0	4.6	4.4	1.7
300	M	6.5	8.0	6.0	6.5	6.8	6.8	7.2	5.4	2.4
	F	6.0	7.5	8.0	6.8	8.0	7.7	7.0	7.3	3.1
360	M	—	—	—	—	—	—	—	5.2	4.8
	F	—	—	—	—	—	—	—	8.0	dead
480	M	—	—	—	—	—	—	—	—	7.0
	F	—	—	—	—	—	—	—	—	dead

Although the onset at 40.5° of protectability (presence of a protectable state) is rapid, its decline is slower. Moreover we may distinguish the relatively fast conversion of B and D to protected states from the much slower conversion of E. The data in Table I show the rapid onset and slower decline of what we may call "quick protectability." Reading across, the ratings fall as the interval (x) increases. As the duration of the first treatment (y) increases, changes in protectability are best seen by reading down the right hand columns. For example, with 6-minute intervals protectability is great after only 10 seconds at 40.5° , while

several minutes at 40.5° are required to progress beyond this stage. Here the protection against crossvein defects is much more dependent on interval length than on pretreatment duration over a large range. Were it not for the previously established distinctions between transient and lasting protection and between the Q_1 's of 1.5 and 1.8, these data would suggest simply a rapid buildup and slow conversion of a rapidly protectable state. As things stand, however, it appears more likely that two states, B and D, exist which are indistinguishable in this experiment.

The disappearance of D coincides with the formation of E, and the decline of rapid protectability thus coincides with the onset of increased morphogenetic sensitivity to temperatures below 39° . Two types of attempt have accordingly been made to measure specifically the $D \rightarrow E$ transition; one measures the decline in protectability, and the other measures the increase in response to a selected temperature below 39° .

We shall first describe the type of experiment which measures the decline in protectability. As we shall see, in one form it is used to measure the disappearance of D, and in another form it is used to measure the disappearance of E. This type of experiment, called a split series, involves a treatment of fixed length split into two parts by an interval. The variable is the placement of the interval. In either case a total exposure to 40.5° (or a nearby temperature) is chosen which ordinarily produces severe crossvein defects but little reduction in survival. An interval at a lower temperature is interposed at each of various times in the treatment. To the extent that the flies are protectable by the interval, the final defect production will be reduced. Figure 2 presents such data. Ten minutes at 37.5° are sufficient to convert a large amount of any D present to C'. B could also be converted to C, but in fact the B is essentially gone after even the shortest first treatment used here. The 10-minute interval at 37.5° will not convert a significant amount of E to a protected state. Thus this split series with a 10-minute interval at 37.5° is used to chronicle the disappearance of D.

An interval at 23° will convert B, D and E to C or C'. In principle, such an interval can be used to monitor the amount of E, which of course remains substantial for some time after the disappearance of D. Figure 3 contains the results of such experiments. Notice by comparing these data to those of Figure 2 that 23° protection is indeed still seen after first treatments too long to be mitigated by a 37.5° interval. To maximize this 23° protection, one would like to use a 60- to 90-minute interval, since conversion of E to C' takes a long time. But intervals of this length force either the early or the late part of the high temperature treatment out of the maximally sensitive period, and so 30 minutes is used as the best compromise.

Although temperatures other than 40.5° can be used for the treatment, the number of events taking place makes it impossible to calculate temperature coefficients for single transitions by this method. Note that teabags are used whenever possible to minimize warmup time; even when the total elapsed time from beginning to end of treatment is too great for a teabag to be used exclusively, it can be used for 20 minutes at 40.5° , after which no protection at 23° is observed, and the pupae are then transferred to vials. The transfer period has no mitigating effect at this time.

To return to D decay, the data in Figure 2 show that, at 40.5°, D is essentially gone after 8 minutes. Further displacement of the interval results in no great increase in ratings, indicating no further loss in 37.5° protectability. At higher temperatures, particularly 42.5°, the overall process of crossvein defect production (appearance of G in amounts exceeding a threshold) takes place so rapidly that one cannot monitor the total disappearance of D. At these temperatures a substantial amount of D is still present when enough G has been formed to cause serious crossvein defects, and even death. This is consistent with the observation that the overall process leading from A to G has a Q_1 of 2.3, while D formation has a Q_1 of 1.8 and the conversion of D to E, though not accessible to exact measurement, seems also to have a Q_1 well below 2. Thus the conversion of a certain amount

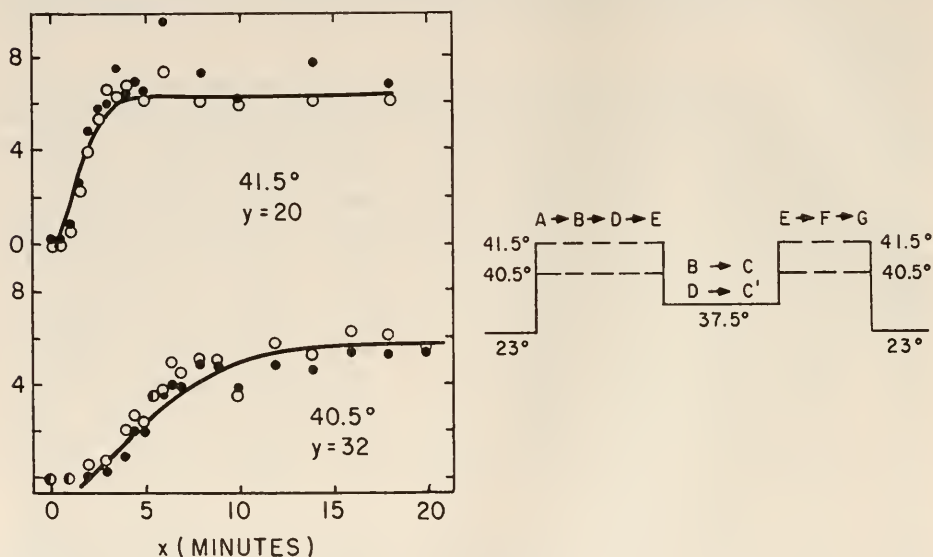


FIGURE 2. Disappearance of D as measured by split series experiment with interval at 37.5° C. Interval begins x minutes after start of treatment; y = total duration (minutes) at 40.5° or 41.5°. As D disappears, the protective effect of 37.5° interval declines; rating thus rises as onset of interval becomes later. Open circles, females; filled circles, males. Temperature sequence and reactions associated with each step diagrammed at right. Dashed horizontal line, variable duration; solid horizontal line, fixed duration.

of D into E will be followed by its subsequent conversion to G before all the D disappears. And this amount of G is enough to cause extreme responses. Why the latter part of the sequence—why indeed the overall sequence—has such a high temperature coefficient will be explained in terms of the D branch point to be discussed shortly.

The other measure of the D → E transition monitors E formation rather than D disappearance; in other words, it follows the increase in sensitivity to lower temperatures rather than the loss of protectability. This kind of experiment involves a variable first treatment at 40.5° (or nearby) and a constant second treatment at a temperature below 39°. The rationale here is that with successively

longer first treatments, the second treatment should become more and more effective in causing crossvein defects because in terms of our scheme, as more E is made, more F can be made at the lower temperature. Thus the response would rise sharply with increased first treatment length, reflecting E formation primarily. After the E is all made (essentially), a further increase in first treatment duration would increase the total response much less, merely in relation to the lengthening of the total treatment; we would see, in a simple case, something approaching a broken

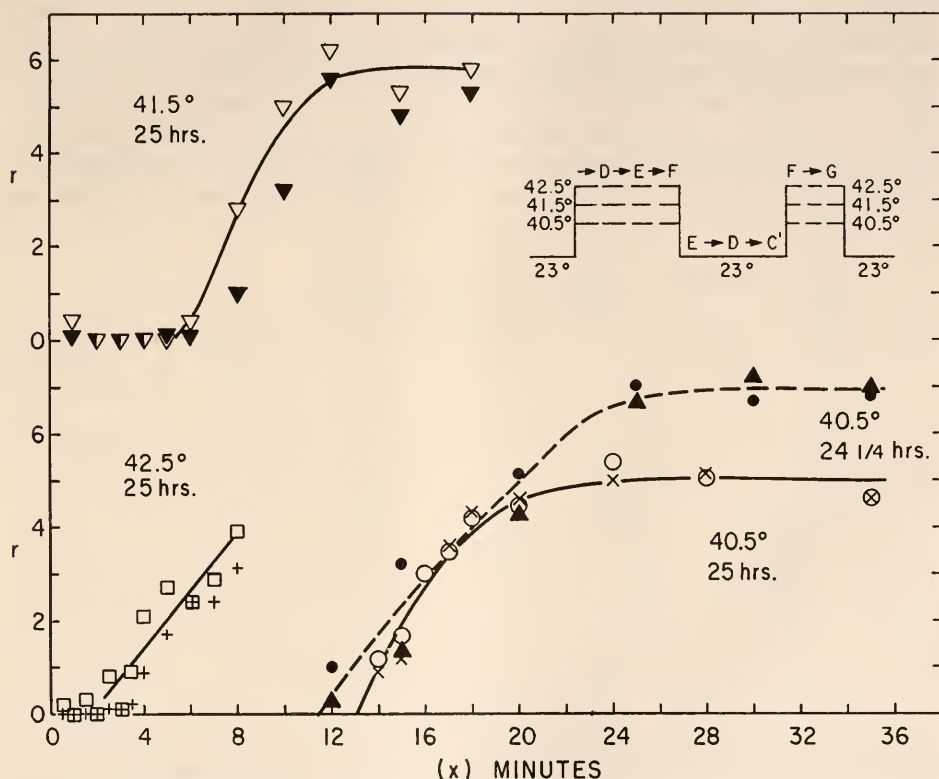


FIGURE 3. Disappearance of E as measured by split series experiment with interval at 23° C. Interval begins x minutes after start of treatment. Total duration at 40.5° = 35 minutes; at 41.5°, 18 minutes; at 42.5°, 8 minutes. As E disappears, the protective effect of 23° interval declines; rating thus rises as onset of interval becomes later. Females: 40.5°, circles; 41.5° and 42.5°, open triangles and squares. Males: remaining symbols.

curve. In practice, as might be predicted (from the apparent overlap of the various transitions, and from the necessarily unequal total treatment durations), the results do not form such a simple pattern. Accordingly, the split series experiments are the experiments of choice. Nevertheless, the broken curve experiments have been of some value (Milkman, 1963), and the data obtained fit the predictions made with our analog computer (Hille and Milkman, 1966).

The conversion of E to F, too, has a measurable course at 40.5° but not at

42.5°. The dose which produces death limits the length of time over which one can follow D decay or E decay. Once more, it appears possible (at 42.5°) to produce a lethal amount of the terminal form before *completing* the transitions in the middle of the sequence. This illustrates the overlaps which we must consider.

The protection of E has been studied in terms of a response *vs.* interval length experiment. Because of the length of time required to protect E, such an experiment runs the risk of extending beyond the sensitive peak period. However, short intervals produce enough information to permit some conclusions. A simple hypothesis is an $E \rightarrow C''$ transition, as was previously proposed (Milkman, 1963). One would expect a great amount of protection with a short interval and a progressive reduction in added protection as interval length increased. An alternative notion of comparable simplicity would involve the reversal of the $D \rightarrow E$ transition, with E going back to D, which will then go to C' , as we have already described. Here one might expect (depending on the actual quantitative characteristics) a lag first, then protection as one increased the interval. The kinetics of protection

TABLE II
Kinetics of E protection; 40.5° (10 min.) + 34.0° (x min.) + 40.5° (y min.).
Data expressed in rating units

x	Males			Females		
	y					
	25	30	37	25	30	37
0	8.0	d	d	8.9	d	d
10	8.6	10.3	(10.0)	8.3	9.7	d
20	7.2	9.2	(9.8	7.0	9.1	d
30	5.4	8.2	8.8	5.8	7.4	8.8
40	4.4	6.4	8.0	4.6	6.3	7.7
50	3.5	4.1	5.3	4.3	5.4	5.1
60	1.8	3.2	3.7	3.0	3.8	4.0

would not be first order, even if each process were a monomolecular event. The experimental results are shown in Table II. They favor the indirect $E \rightarrow D \rightarrow C'$ path, since there is a lag of 10 or 20 minutes, after which protection increases linearly with interval. Accordingly, we presently believe that protection of E involves its conversion back to D and then to C' .

After about 20 minutes at 40.5°, essentially all the A has gone either to F or to the protected states. No further protection is possible. This is true, not only for 25-hour pupae but for younger and older ones as well. The evidence for this comes from a comparison between two kinds of age-response curves. The first relates responses to 35 minutes at 40.5° with respect to pupal age. The second deals with split treatments: 20 minutes at 40.5° are administered at a given age, and the rest at 25 hours, the age of peak sensitivity (Milkman, unpublished). These curves are essentially parallel (the split treatment curve shows greater response, due to the contribution of the treatment at peak sensitivity), but treatments much shorter than 20 minutes at any age, followed by room temperature, confer protection.

Since the 20-minute first treatment is the significant variable when administered at or before 25 hours, and since the $F \rightarrow G$ transition is not important until later, it appears that one or more of the early steps is age-dependent, resulting in a variable proportion of A going to a protected state in the first 20 minutes.

After 25 hours, however, the latter part of the treatment is the important variable, indicating either that $F \rightarrow G$ slows down with increasing age, or that the effect of G production on posterior crossvein formation decreases with time. The cross-

TABLE III

Results of treatment completion at lower temperatures; first treatment: 25 minutes at 40.5°

Second treatment		r	
Temperature (°C.)	Duration (min.)	♂	♀
32.5	250	10.4	9.9
	200	8.8	8.5
	150	6.2	6.5
	100	4.3	6.3
	50	2.7	4.4
	40	1.6	3.2
31.5	240	8.8	8.7
	120	3.8	5.6
30.5	240	7.9	7.1
	150	4.4	6.3
	120	2.5	5.1
	90	2.5	4.5
	60	1.8	4.1
29.5	240	7.9	7.9
28.5	240	4.5	6.7
27.5	240	4.0	5.0
26.5	240	2.6	4.5
24.5	240	0.5	1.5
22.5	240	0.1	1.6
18.5	240	0.0	1.1

vein is being laid down at this time (Mohler and Swedberg, 1964; Waddington, 1940).

The last step in the sequence, $F \rightarrow G$, proceeds much more slowly than the others, and it has been the easiest to study with respect to its temperature coefficient. Merely by treating the pupae for 25 minutes at 40.5°, one reaches a stage where essentially only this reaction is going on. Previous data (Milkman, 1963) on the amount of subsequent time at various temperatures needed to produce a certain

average defect have indicated a Q_1 of 1.4–1.5 down to 33.5°. Further experiments have extended this relationship down to 26.5°, below which temperature the durations required would be excessive. The new data are given in Table III and illustrated, together with the previous data, in Figure 4. These results, incidentally, point up the importance of controlling temperature after treatments.

Although the temperature coefficients are accessible to study, the actual rate of the process is not easy to describe in terms other than average amount of defect produced per unit time. If $F \rightarrow G$ is a first order process, it is very slow indeed, since the relationship between dose and response has a linear appearance. Only

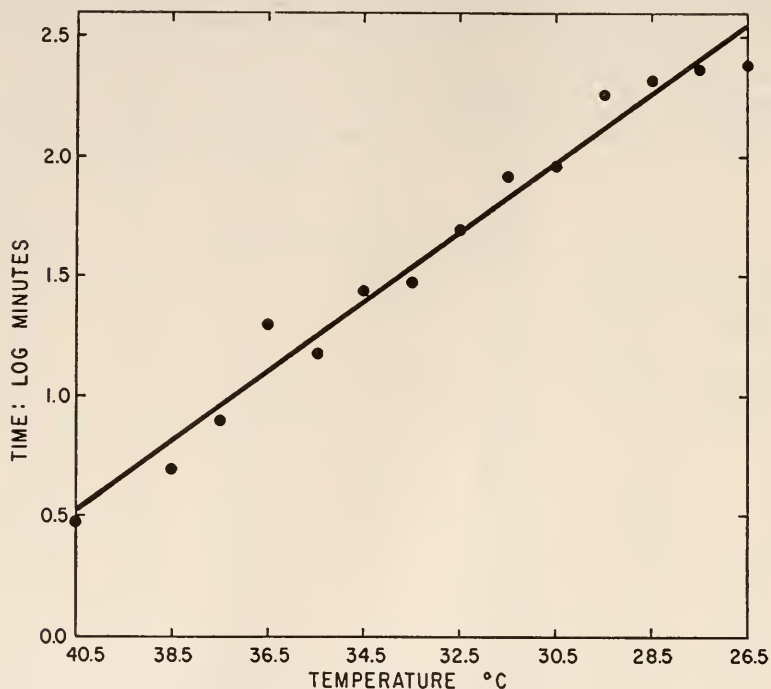


FIGURE 4. Determination of the temperature coefficient of the $F \rightarrow G$ transition. Ordinate: log duration at a given temperature which, when added to a 25-minute exposure to 40.5° C., produces a criterion response (sum of male and female ratings = 7). Visually plotted line corresponds to $Q_1 \cong 1.4$.

the early part of a first order reaction appears linear. This, in turn, would imply that very little loss of the hypothetical functional protein would lead to morphological defects and that very little further loss would lead to death. This is not unreasonable in a living organism, particularly since we have sought out this developmental process on the basis of its sensitivity. There is no present basis for further speculation, however.

The measurements of overall temperature coefficients at higher temperatures, difficult in vials because of the significant warmup period, have been extended through the use of teabags. The durations at temperatures from 40.0° to 42.5°

required to produce a criterion response (sum of male and female ratings = 6.0) have been compared and are plotted in Figure 5. These data yield an overall Q_1 of 2.0, somewhat lower than the value previously obtained (2.3) but still higher than the Q_1 for any individual step.

The paradox thus remains that the overall Q_1 appears to be between 2.0 and 2.3, while the individual steps appear to have Q_1 's no higher than 1.8, with the Q_1 of the slowest reaction, $F \rightarrow G$, being 1.4–1.5. It was proposed (Milkman, 1963) that the overall Q_1 represented a product of two kinds of temperature functions. One, the temperature coefficient of the rate, is familiar, but the other is less obvious. It is the proportion of a form proceeding to one of two possible subsequent forms. We illustrate with D because it appears in practice to be the only relevant case *at*

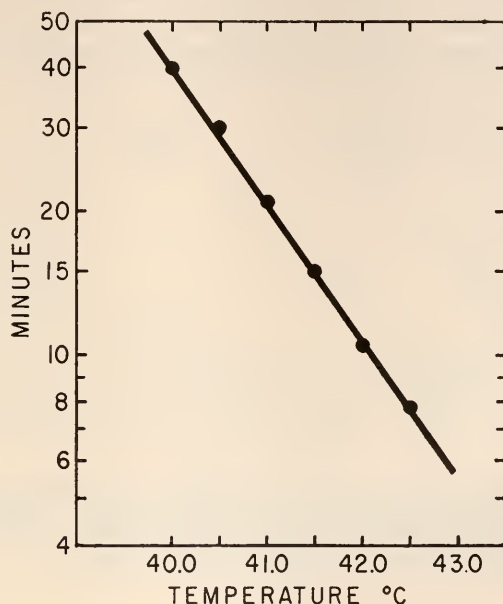


FIGURE 5. Determination of the overall temperature coefficient. Ordinate: duration at a given temperature which produces a criterion response (sum of male and female ratings = 6). Visually plotted line corresponds to $Q_1 = 2.0$.

the temperatures we are considering. Since D can go to C' and also to E, and since $D \rightarrow C'$ has a temperature coefficient of 1 (is temperature-independent) and $D \rightarrow E$ has a temperature coefficient in the neighborhood of 1.7, we can imagine a low temperature at which all the D goes to C', a high temperature at which all the D goes to E, and an intermediate range over which the proportion of D going to E is a sensitive function of temperature. In a first order system, the rate of formation of F is $k_{EF} [E]$, and since k_{EF} and $[E]$ are both temperature-dependent, the rate must depend on the product of these temperature-dependent functions. Similarly the rate of formation of F must have a two-factor temperature dependence even if we treat the pupae at a single temperature from beginning to end; and dG/dt must share this dual dependence. Thus the overall temperature coefficient,

$Q_1 = 2.0$ – 2.3 , could be factored into the product of 1.4 – 1.5 ($Q_{1\text{FG}}$, $F \rightarrow G$ being the slowest reaction in sequence) times 1.3 – 1.6 , which we attribute to the temperature dependence of the proportion of D going to E.

Experimental support for this hypothesis comes from examining the response to 39.5° of pupae which have largely reached the F stage at one of a number of experimental temperatures. Clearly the rate constant of $F \rightarrow G$ at 39.5° , k_{FG} , is fixed. But if the total amount of E made, and therefore the total amount of F made, is temperature-dependent, then the dose-response relationship at 39.5° will depend upon the temperature at which the early part of the treatment was given. The results are illustrated in Figure 6, supporting qualitatively the hypothesis that the nature of the Q_1 of 2.0 – 2.3 is compound. The slopes of the dose-response curves show the relationship between rate of G synthesis at 39.5° and the temperature at which E was made. The increase in slope is about 1.3 per degree, within the range 1.3 – 1.6 predicted crudely here, and in good quantitative agreement with

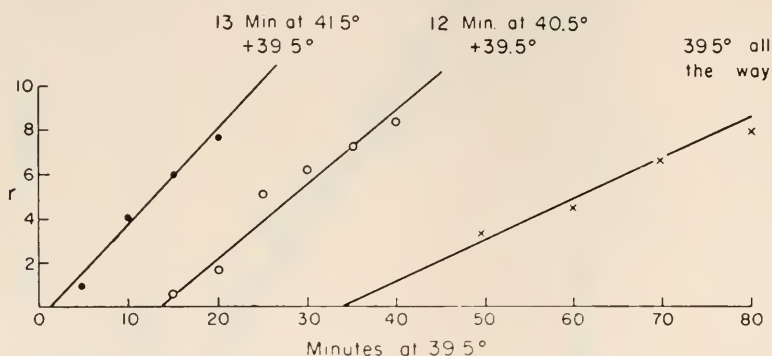


FIGURE 6. Dependence of dose-response relationship at 39.5° upon temperature of first part of treatment. Examples selected for purposes of effective display. Data from additional experiments at 41.5° and 40.5° where first treatments have different durations show similar slopes. Lines drawn from computer calculations (Hille and Milkman, 1966).

our computer predictions, which can and must take a number of additional factors into account (Hille and Milkman, 1966).

It is noteworthy that, no matter what the assortment of treatments within the present range (and specifically excluding 4 hours at 37.5° reported previously [Milkman, 1963] and described as not capable of inclusion in the present scheme), an average defect rating of 9 or more is always accompanied by high mortality and 11 is virtually unattainable, apparently because of death. It is possible to protect against crossvein defects so that death comes first; but it is not possible to produce extreme crossvein defects and yet retain good survival. Thus it seems that the hypothetical protein involved in crossvein formation is also vital—presumably elsewhere in the organism—and there it has a slightly greater reserve which can be destroyed before measurable effects appear. When death occurs in pupae protected against crossvein defects, it requires a longer treatment than usual and is presumably due to an event which ordinarily is preceded by death due to the destruction of the crossvein protein.

One can calculate first order rate constants for each of the transitions, on the basis of the relationship $k = 0.69/t_{1/2}$, where $t_{1/2}$ is the time for the reaction to proceed halfway. One might expect tertiary structure transitions in proteins to have first order kinetics, since many denaturation processes do. The rate constants have thus been calculated for all the processes that reach completion (essentially). For $F \rightarrow G$, which comes nowhere near completion, not even enough curvature can be seen in its time curve to permit a calculation of a rate constant. This fact does set an upper limit, though, which enables us, rather speculatively, to set the rate constant in the range of 0.01. Table IV lists all the first order rate constants.

An heuristic aspect of setting rate constants in this way—based on the individual processes—is that we can try to see whether in concert they are consistent with a

TABLE IV
*First order rate constants for the individual steps**

Transition	Rate constant (min. ⁻¹) at 40.5°C.
A $\rightarrow$ B	10
B $\rightarrow$ C	0.15
B $\rightarrow$ D	4.0
D $\rightarrow$ C'	0.15
D $\rightarrow$ E	0.10
E $\rightarrow$ F	0.15
F $\rightarrow$ G	0.01**

* Estimates are given for the forward steps only; for complete list of constants, see Hille and Milkman, 1966.

** Very rough estimate; see text.

wide variety of results at various single temperatures and combinations of temperatures. Such an analysis is to be found in another paper (Hille and Milkman, 1966).

DISCUSSION

The work described in this paper is largely confirmatory and raises no new issues (*cf.* Milkman, 1963). The minor changes in the overall scheme are the dropping of one tertiary state (C'') and the introduction of a reverse arrow between D and E. It appears from the body of information now accumulated that the various transitions are valid, both in themselves and in relation to the others. Moreover the practical limit seems to have been reached in terms of the experimental dissection of steps in the overall process. There are certain temperatures at which some transitions are prominent; the $E \rightarrow F$ transition, however, is not resolvable over a broad enough range to calculate a temperature coefficient.

The notion that the transitions are related in a sequence undercuts the calculation of an original amount of A, relative to a threshold and relative to crossvein defect rating units, by the simple extrapolation of G formation. Similarly, the branch ratio at D cannot be considered in isolation, as one factor of a two-factor product, if one is to systematize the several events and their outcome over a broad temperature range. Now that the best estimates obtainable from these

experiments are at hand for the time course of each transition, and since the postulated tertiary structure changes suggest first order kinetics and easily calculated rate constants, it is of theoretical and practical value to place the calculated values in the postulated scheme. By so doing, one can attempt, with the aid of an analog computer, to predict the results of an array of simple and complex treatments that would otherwise be difficult to approach. This is done in another paper (Hille and Milkman, 1966).

Very little conclusive information has been added lately with respect to the mechanism of temperature adaptation and other temperature effects, although such effects are being studied in a number of theatres, with findings that are interesting in themselves and as leads (Ushakov, 1964; Prosser, 1966). Since Northrop's work (1920) on temperature adaptation in *Drosophila*, which was conveyed from one generation to the next *via* the egg, the observations on this animal and others have been intriguing but neither explained nor thoroughly generalized. Mohler's (1965) finding that not all parts of the posterior crossvein respond identically to high temperatures in flies of certain genotypes makes it clear once again that the present scheme holds in a restricted theatre at best.

The idea of tertiary structure change has little direct experimental support. One of us (R. M.) has attempted using both *Drosophila* extracts and pure proteins (bovine serum albumin, hemoglobin) to demonstrate heat-induced deviant tertiary structure states as expressed by altered migration rates in acrylamide gel disc electrophoresis. After numerous failures, recent efforts with larval extracts treated at 55° have been successful (Milkman, in Prosser, 1966). These experiments are still preliminary and we know of no similar work; yet it is logical at present to allow for the transition of some proteins among various stable or meta-stable tertiary structure states, rather than restricting them to a single-structured condition whose only alteration would be denaturation to a disordered state. For further discussion of this subject, see Milkman in Prosser (1966).

Finally, it should be emphasized that the collected evidence presented here strongly supports a single branched sequence, whatever the fundamental mechanism, because of the indissociability of the steps from one another and the impossibility of rearranging them.

The assistance of Mary Ann Cady, Tonja Knapp and Maren Brown is acknowledged with thanks.

This work was supported by National Science Foundation Grant G-24023.

SUMMARY

1. A previously presented scheme unifying a variety of high temperature effects on day-old *Drosophila melanogaster* pupae has been confirmed substantially by a large body of new data. Several minor modifications have been made.

2. New experiments confirm the validity of analyzing heat-induced temperature adaptation over a several-hour period at the end of the first day of pupal development.

3. Several new types of experiments have been described which involve combinations of exposures to more than one temperature and which provide information on the transitions between several intermediate stages in the scheme.

4. A double temperature-dependence is demonstrated in which both the amount of a precursor and the rate of its conversion vary with temperature in a particular range. In this range, the temperature coefficient of crossvein defect induction is the product of two components. This property inheres in branched pathways.

5. Rate constants and temperature coefficients have been calculated for the individual steps on the basis of data from specific experiments.

6. The scheme incorporates the information from a large array of detailed experiments and is capable of generalization over a broad temperature range.

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A QUANTITATIVE DESCRIPTION OF SOME TEMPERATURE EFFECTS ON DROSOPHILA

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The appearance of the posterior crossvein of the wing of adult *Drosophila* is a delicate measure of physicochemical processes underlying wing vein development during early pupal life. Previous studies of the disturbance of posterior crossvein formation by temperatures from 30° C. to 42.5° C. (Milkman, 1961, 1962, 1963) have led to the outlining of a complex hypothesis which seemed capable of explaining a large number of experimental results (Milkman, 1963). It is the purpose of this paper to cast the hypothesis in a completely quantitative form and to show that the predictions of the model do indeed agree with published and hitherto unpublished experiments.

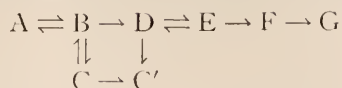
The experimental background of these studies starts with the basic observation that heat damage to the development of the posterior crossvein is a threshold phenomenon. At temperatures from 39.5° to 41.5° the time to reach a threshold response, or any more severe disturbance, decreases by a one-degree temperature coefficient (Q_1) as large as 2.3. Subthreshold effects are readily revealed by additional heat treatments to consist of a series of related processes which precede the process leading to damage. Nearly threshold pretreatments are definitely damaging as they are additive with a second treatment. Briefer pretreatments, however, would in certain conditions lead to a rapid temperature adaptation which protected the pupa against the damaging effects of additional treatments. In some cases the protection was transient and in others it persisted for many hours. The wealth of effects emerging from these experiments led to the original kinetic scheme (Milkman, 1963). It was suggested that high temperatures induced a sequence of tertiary structure changes in some hypothetical protein required for crossvein formation in the pupa. One could explain the various effects of pretreatments by the properties of the predominant tertiary structure state remaining after the pretreatment. The sole and yet compelling evidence that a protein's tertiary structure was being changed was the very high temperature coefficient of some of the individual steps.

POSTULATES

The full details of the original scheme can be incorporated into four postulates which may be regarded as the hypothesis to be demonstrated by this paper:

1. Normal development of the posterior crossvein requires a protein A within a few hours after a biological age equivalent to 25 hours of pupal development at 23° C.

2. At elevated temperatures A is changed to states B, C, C', D, E, F and G as in the reaction sequence below:



The individual steps of the conversion, represented by arrows, are first order in the reactant and may be assigned rate constants and temperature coefficients.

3. The algebraic sum of the amounts of protein in all the states except G linearly determines the ultimate length of the posterior crossvein within a defined range. Thus defects result from treatments which produce G, the terminal inactive state.

4. The adjustable parameters of the model: rate constants, temperature coefficients, initial amount of A, and the function relating posterior crossvein length to the sum of the effective states, are themselves functions of the age, raising temperature, sex, and genome of the organism.

MATERIALS AND METHODS

Because of postulates 1 and 4, in this paper we evaluate the parameters only for female Oregon R *Drosophila melanogaster* 25-hour pupae raised at 23° C. This age happens to be the time of maximum heat sensitivity of posterior crossvein formation (Milkman, 1961, 1962).

The equations of the model were solved on a ten-amplifier Donner 3500 analog computer using standard techniques for linear systems. Initial conditions were set with a Tektronix 161 pulse generator. The time course of the solutions was photographed on Polaroid projection film from the face of a Tektronix 502 oscilloscope. The curves were enlarged on graph paper by tracing the projected image under an enlarger. We often took advantage of the additivity of solutions of linear systems when curves for experiments involving split treatments were to be calculated. In a two-temperature experiment, for example, the quantities of all the intermediates after the first treatment were calculated in a straightforward manner and then the separately calculated fates of each intermediate in the second treatment were added to get the final solution. The maximum error was less than 5%. This error will be seen to correspond to an error of less than one crossvein rating unit in the predictions.

The fruit flies used in these experiments were a highly inbred strain of Oregon R *Drosophila melanogaster* raised by standard techniques in an incubator at 23°. Pupae were placed in shell vials within one hour after puparium formation, to be aged in a 23° regulated water bath. Depending on the duration of treatment, high temperature heat treatments were started sometime after the 24th hour of development and were completed by the 26th hour. Unfortunately, even during this period, the response of the flies changes sufficiently rapidly to affect the agreement between experiment and predictions (Milkman, 1961, 1962). Short experiments whose total treatment could be given close to the 25th hour were therefore considered to be of the greatest importance. The high temperature water baths were regulated to within $\pm 0.05^\circ$, but their absolute temperature can have differed by $\pm 0.15^\circ$ from one year's experiments to the next. This variation of temperature would lead to a variation in experimental data approximately equivalent to a 10% change in the time axis. For all short treatments the pupae were in a teabag. Teabags or

vials were used in longer experiments. While the results of identical treatments in teabags and vials are different at times, we have made no effort to distinguish them in this paper. We have also ignored any possible warm-up time for glass vial treatments. At least five days later, after the flies had emerged, their posterior crossveins were rated on a scale from zero (normal) to twelve (none remaining) according to the total length of posterior crossvein remaining on the two wings and regardless of how the remaining fragments were distributed. The ratings (r) of about 50 flies were averaged for each data point. The details of these methods have been published before (Milkman, 1961, 1962). Almost all of the data used here have been published already (Milkman, 1962, 1963; Milkman and Hille, 1966).

The figures of this paper contain points, lines, and a summary of the temperature sequences used. The points are always experimentally determined data points referring to large numbers of flies. The lines are theoretically calculated using the explicitly stated postulates, the rate constants, and the conversion factor between G units and crossvein rating units. There are no additional adjustable parameters, so that to the degree that the lines and dots in all examples agree, the unified scheme is satisfactory. The temperature sequences have been indicated diagrammatically by a series of rectangular line steps. If the duration at a particular temperature is variable, the horizontal line has been dotted; otherwise the line is solid.

RESULTS

To explain how our hypothesis fits experimental data we must first examine certain mathematical properties of the model system without reference to any experiments. According to postulate 2, first-order differential equations for the rate of change of each state may be written in the conventional form of chemical kinetics:

$$\frac{dA}{dt} = -k_{AB}A + k_{BA}B$$

$$\frac{dB}{dt} = k_{AB}A - (k_{BA} + k_{BC} + k_{BD})B + K_{CB}C$$

$$\frac{dC}{dt} = k_{BC}B - (k_{CB} + k_{CC'})C$$

$$\frac{dC'}{dt} = k_{CC'}C + k_{DC'}D$$

$$\frac{dD}{dt} = k_{BD}B - (k_{DC'} + k_{DE})D + k_{ED}E$$

$$\frac{dE}{dt} = k_{DE}D - (k_{ED} + k_{EF})E$$

$$\frac{dF}{dt} = k_{EF}E - k_{FG}F$$

$$\frac{dG}{dt} = k_{FG}F$$

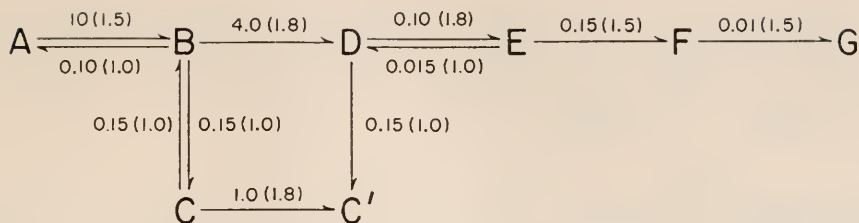


FIGURE 1. The parameters of the model. The first number by each arrow is the first order rate constant in min^{-1} for that step. The second number, in parentheses, is the one-degree temperature coefficient, Q_1 , of the rate constant.

This linear system of simultaneous differential equations may be solved analytically to give an answer in closed form, but this type of solution is impractical because of the tedious calculations required to make quantitative predictions. For this reason we used an analog computer, an ideal instrument for linear systems, to see solutions displayed directly on an oscilloscope screen where the effect of any adjustment in the parameters was immediately observable.

Figure 1 shows the rate constants estimated for 40.5° . Figure 2 is a photograph of a solution using these rate constants and starting with 100 units of A and zero units of all other states. In a fraction of a minute A is gone. The subsequent intermediates rise to a peak and fall again until by 25 minutes the only reaction proceeding at a significant rate is the conversion of F to G. It will be a helpful rule to remember that even in this complicated system most reactions will have half-times given in minutes by 0.7 times the reciprocal of the rate constant k . In the presentation we shall speak loosely of the time when a certain reaction is complete. We shall really refer to the time when the reaction is 80% to 90% complete.

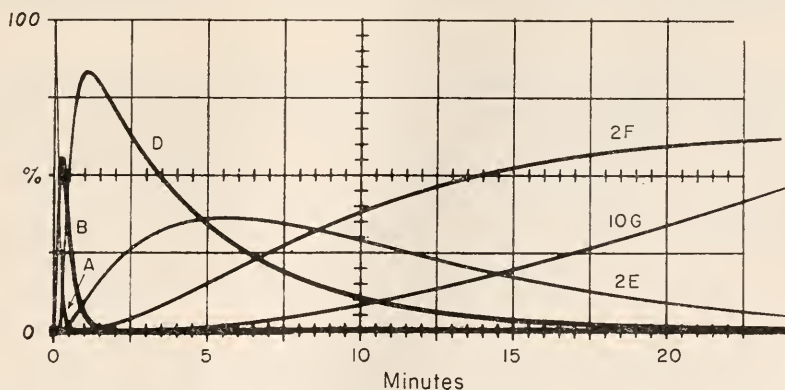


FIGURE 2. The time courses of the states of the model at 40.5° photographed from the face of the oscilloscope. The solution was started with 100 units of A (four boxes on the reticle) and zero units of all other states. As indicated, some of the curves are recorded at higher gain for better resolution. C formation is negligible. C' formation is 60 units at 20 minutes. Neither state is recorded here. At other temperatures the temporal relationships are different.

As the temperature is raised many of the reactions of the model speed up and the intermediate states come and go more rapidly. Figure 1 gives one-degree temperature coefficients (Q_1) to be used in the following relation to calculate the rate constants at any temperature (T) :

$$k(T) = k(40.5^\circ) Q_1^{T-40.5^\circ}$$

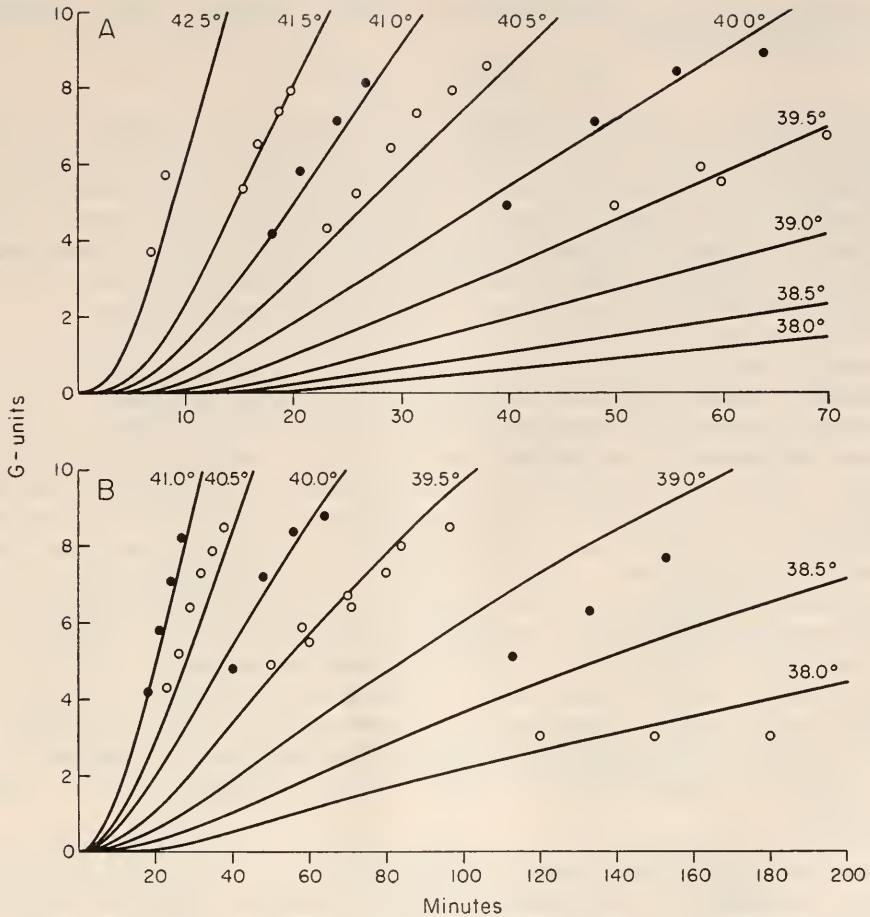
A consequence of the general speeding up with temperature may be seen in Figure 3 where the family of solid curves represents the time course of G formation at different temperatures. These curves behave as though they were generated by a process having a Q_1 greater than 2.0. This is surprising at first because Q_{1FG} is only 1.5 and no other reaction has a Q_1 higher than 1.8. The extra multiplicative factor of more than 1.3 is explained as follows:

There are two terminal states in the model, C' and G. In general we may say that the more C' is made, the less material can ever become G and the slower is G formation at any time. Thus we could call C' a "protected" state because its formation prevents the formation of G, the damaged state. At 40.5° the pattern of rates of the reaction favors the production of C' over the production of G. At higher temperatures there is a different pattern and more material is destined for G. It is this temperature-dependence of the amount of G to be formed that provides the additional factor which accounts for the very high Q_1 of G formation.

The amount of C' formed depends on four important pathways of protection: directly from D, directly from C, indirectly from B *via* C, and indirectly from E *via* D. Each of these assumes importance at different temperatures. Let us first consider the direct route from D. Some D goes to E and some to C'. Thus D may be said to be at a "branch point." The velocities of E and C' formation from D are in the same proportion as the rate constants k_{DE} and $k_{DC'}$. We therefore define the concept of "D branch ratio" as k_{DE} divided by $k_{DE} + k_{DC'}$. In words it is the fraction of D going to E, but we must bear in mind that some of the E produced may be returning to D at the same time. Similarly the B, C, and E branch ratios may be defined as in Figure 4. Larger branch ratios mean less C' and more G formation. We shall see presently how they are useful.

One rate constant in each branch ratio is temperature-sensitive and one insensitive, making the ratio a strong function of temperature, as can be seen in Figure 4. Notice that the only significant contribution to C' formation in the range from 38.5° to 42.5° is at the D branch point and that its variation with temperature is sufficient to give the required factor of 1.3.

If the reactions of the model are interrupted after some time and temperature when there is a large amount of D, to be continued at a temperature at which the D branch ratio is very small, then almost all the D will be channelled to C'. In this case there is protection against G formation at high temperatures. Similarly with E, if the temperature is lowered to where both the E and D branch ratios are small, E can be diverted *via* D to C'. Protection from B is only slightly more complex in that the B and C branch ratios are never simultaneously very low. Therefore a low temperature is first required to make C followed by a high temperature to make C'. Because C readily forms C' at high temperatures, we shall also use "protected" to describe C. Thus the curves of Figure 4 are useful for selecting temperatures at which particular pathways of C' formation will be operant.



FIGURES 3A AND 3B. The time courses of G formation at various temperatures starting with 100 units of A at zero time. In this and all figures after Figure 4 the solid lines are the expectations of the model and the points are the average response of a large number of female flies. The two always reflect the linear relation between crossvein rating units (r) and G formation derived in the text. The points in this figure are from Figure 1b of an earlier paper (Milkman, 1962) plus a few unpublished data. Except at 39.0° and below, the lines run close to the data points. The three points at 39.0° are below the line and the three points at 38.5° are down at 3 G units, corresponding to no crossvein defects ($r = 0$). No points are shown from 38.0°. Whole degree points, filled circles. Half degree points, open circles.

Applying the model

We are now prepared to apply the model to experiments. The first step is to determine the linear relation between crossvein rating units and G formation as in postulate 3. We shall choose the original (Milkman, 1961) dosage-response experiment at 40.5° as our standard. The threshold for defect production was around 20 minutes and average ratings of 10.0 were produced by 40 minutes. Thus the range of G values from 20 to 40 minutes, namely from 3.0 units to 8.5 units,

corresponds to crossvein ratings from 0.0 to 10.0. The linear scale is established by the following relation:

$$\text{rating} = (\text{G units} - 3.0) \times 1.8$$

On this basis the experimental dosage-response data points previously reported (Milkman, 1961, 1962) and some unreported data have been drawn in Figure 3. The model fits the data fairly well. We believe that the actual ratings at low temperatures are less than the predictions because the lengthy treatments required necessarily pass out of the period of maximum heat sensitivity which we have chosen to describe. We have already explained why the model with no Q_1 larger than 1.8 fits dosage-response data with a much higher temperature coefficient.

Uninterrupted single-temperature treatments cannot reveal the many postulated intermediate states. Two- and three-part treatments form the crux of the basis of the model. Many experiments were based on the following reasoning. The pre-treatment at an elevated temperature such as 4 minutes at 40.5° brought the pupae to some combination of intermediate states which in our example can be read from Figure 2. Then an interval at a lower temperature diverted material at the three branch points from the path to G to the protected states C and C'. The interval was followed by a treatment at an elevated temperature designed to assess how much material had been diverted at the branch points. Suitable selection of the interval temperature and duration permitted a partial resolution of the three branch points. Such experiments established a schedule for the reactions at 40.5° published in a previous paper (Milkman, 1963, Table VI), where the reactions $A \rightarrow B$, $B \rightarrow D$, $D \rightarrow E$, and $E \rightarrow F$ were shown to be near completion at 10 seconds, 30 seconds, 5 minutes, and 12 minutes, respectively. This schedule sets stringent conditions on the values of the rate constants. In Figure 2 the same four conversions in the model are half complete by 4 seconds, 15 seconds, 3 minutes, and 10 minutes, respectively.

We have shown that our model agrees both with the overall dosage-response experiments and with the general schedule at 40.5° . Now we shall examine in detail its capabilities in more specific experiments designed to test the individual rate constants and temperature coefficients over a wide range of temperatures.

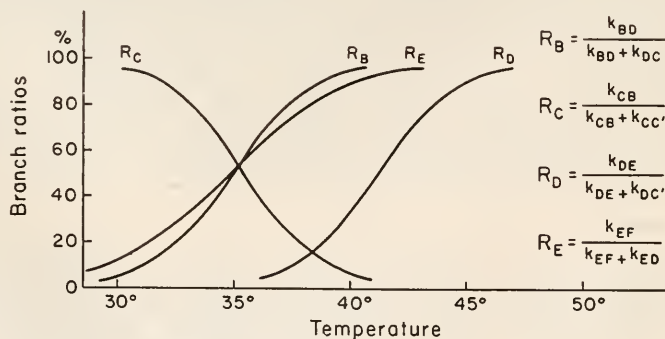


FIGURE 4. The branch ratios as a function of temperature. The ratios are defined on the right and explained in the text. In general, the larger the ratio at a branch point, the more material can get from that branch point to G.

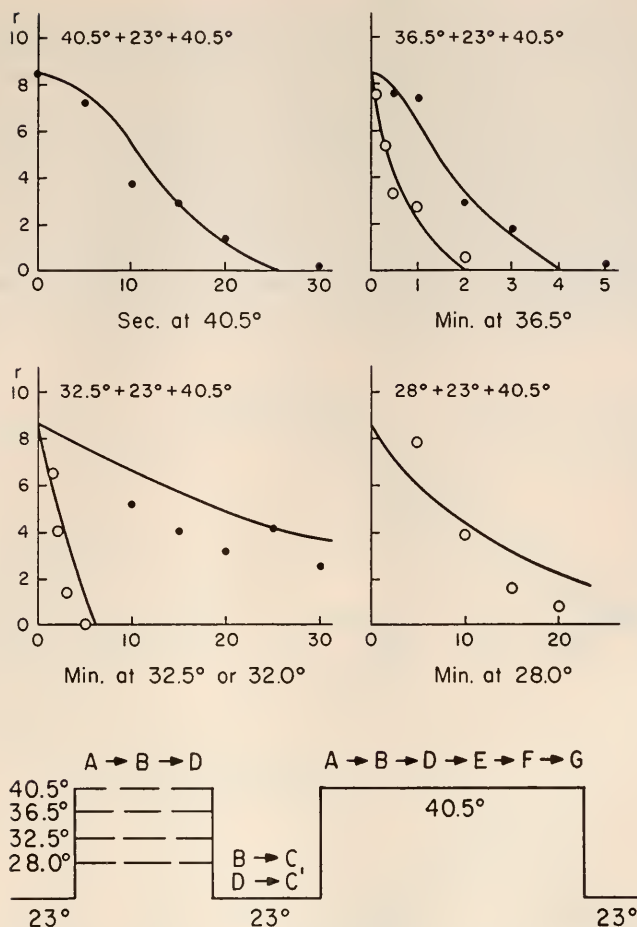


FIGURE 5. Transient and lasting rapid temperature adaptation at different temperatures. The pretreatment temperature and duration are indicated. Treatments were 37 minutes at 40.5° . Transient adaptation (open circles) was achieved with a 5-minute interval at 23° . These data from previously published experiments (Milkman, 1963, Table II). Lasting adaptation (solid circles) used a 60- to 90-minute interval, depending on duration of pretreatment (at 24 hours). These data are unpublished but similar to published experiments (Milkman, 1963, Table IV) where pretreatments started at 21 hours. The ratings decrease as protection increases.

B, C, and D formation

The first three states are normally in an equilibrium mixture of 75% A, 6% B, and 19% C at 23° . When the temperature is raised, k_{AB} is increased, more A goes to B, and the equilibrium shifts, B and C becoming re-equilibrated in about 10 minutes. If the temperature is so high that the B branch ratio is large, then most of the B will continue to D, never to equilibrate with C. Returning the temperature to 23° at any moment will let the $B \rightarrow C$ reaction equilibrate to one part B to three parts C in 10 minutes. At the same time B is returning to A until after 40

minutes the original 23° equilibrium ratios are nearly restored. The actual amount of material remaining at A, B, or C will be reduced if some D has formed at the high temperature. Any D existing on return to 23° will be converted to C' in 10 minutes.

These properties of the model explain the phenomena of transient and lasting rapid temperature adaptation which have been described earlier (Milkman, 1961, 1962). Figure 5 shows the response to 37 minutes of 40.5° after the indicated pretreatments and an interval at 23°. The 5-minute interval used to demonstrate transient rapid temperature adaptation (open circles) was sufficient to bring approximately half the B and half the D to the protected states C and C'. As the major factor in leading to predictability in this experiment is the increased $A \rightarrow B$

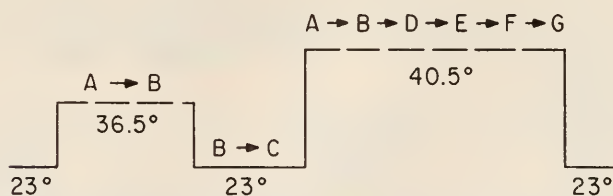
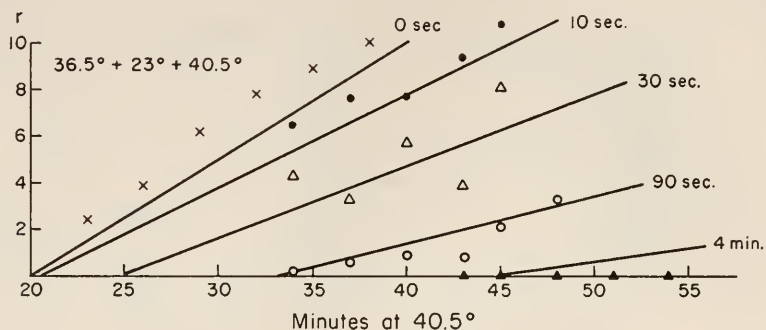


FIGURE 6. Transient rapid temperature adaptation. Dosage-response at 40.5° following pretreatments at 36.5° with a 5-minute interval at 23°. As A is converted to B, the 23° interval diverts more B to a protected state. The protection is manifested as a change in both intercept and slope. Unpublished data.

reaction, we take the agreement of the data (open circles) with the theory (curves) over an 8.5° range to confirm our choice of k_{AB} and Q_{1AB} . The long interval used to demonstrate lasting rapid temperature adaptation (solid circles) was sufficient completely to protect D as C' and to reverse the formation of B and the protected state C by re-establishing the 23° equilibrium ratios. The agreement between data and theory over 8° confirms the choice of k_{BD} and Q_{1BD} . A second kind of experiment showing that transient rapid temperature adaptation leads to the predicted lowering of the 40.5° dosage-response in slope as well as response to 37 minutes is illustrated in Figure 6. This experiment suggests that rather than just creating a delay in G production, protection diverts material from the pathway to G as we have postulated.

E and F formation

As can be seen from the D branch ratio in Figure 4, protection of D by C' formation is nearly complete at temperatures below 38°. This step takes about 10 minutes ($k_{DC'} = 0.15 \text{ min.}^{-1}$). Protection of E *via* D formation is slower ($k_{ED} = 0.015$) and is not large above 35°. Therefore a 10-minute interval at 37.5°, by selectively protecting D and not E, serves to measure the time course of the D to E transition. A time course of this transition is recorded in Figure 2. Figure 7 shows an experiment where a 10-minute interval at 37.5° was intercalated between 40.5° treatments totalling 32 minutes (open circles) or between 41.5° treatments

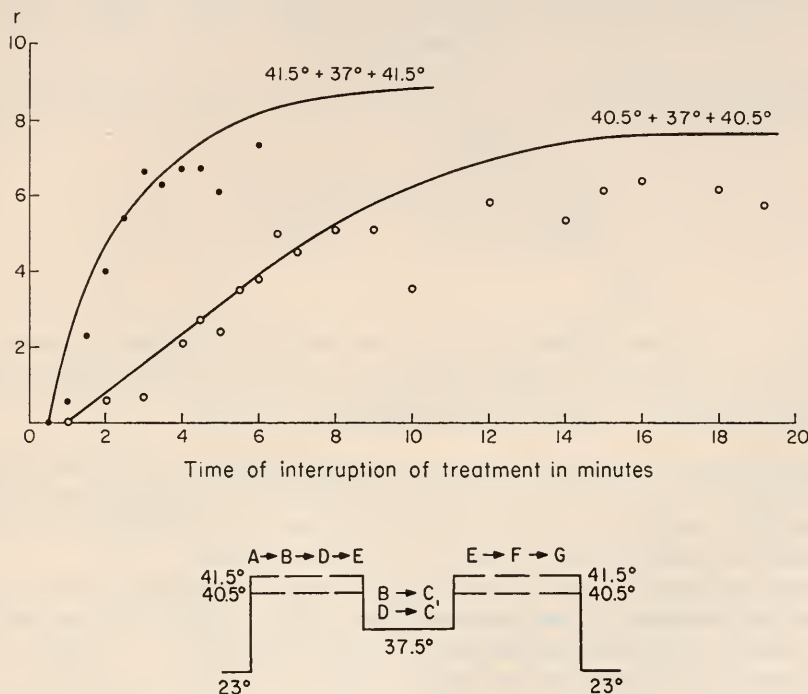


FIGURE 7. The disappearance of D. The response to a 20-minute treatment at 41.5° (solid circles) or a 32-minute treatment at 40.5° (open circles), each with a 10-minute interval at 37.5° intercalated at one of various times (time of interruption). As D is converted to E, the interval at 37.5° becomes less effective in lowering the rating. Data from Figure 3 of Milkman and Hille (1966).

totalling 20 minutes (solid circles). The time and rate of decrease of protectability as D goes to E confirms our choice of k_{DE} and Q_{IDE} .

Because the preceding experiment covered only a 1° range, it is desirable to determine Q_{IDE} over a wider range. This can be done by designing experiments to test the D branch ratio at different temperatures, for, as we have discussed, the temperature dependence of this ratio is a consequence of Q_{IDE} . The branch point ratio may be measured by first exposing pupae to different high temperatures until all the D has been converted to E and C'. Then the slopes of subsequent dosage-

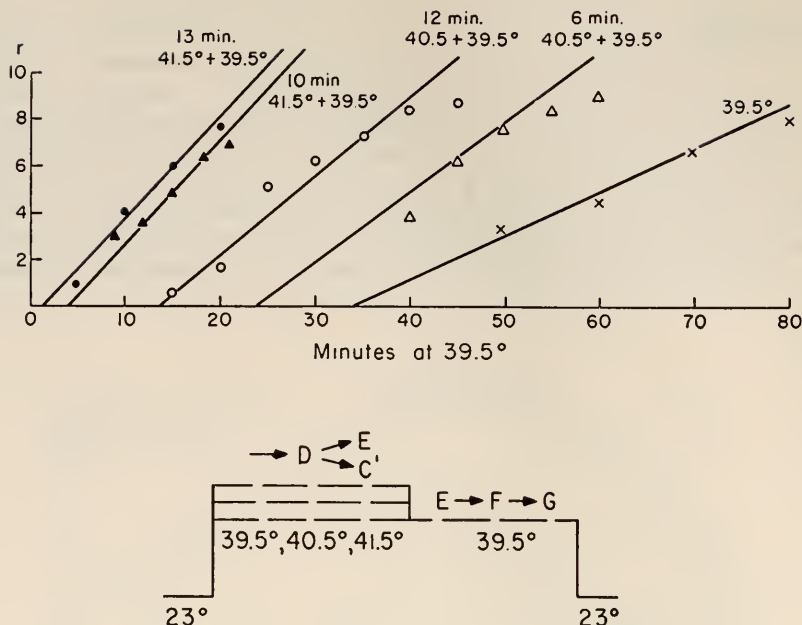


FIGURE 8. Response to 39.5° immediately after exposure to indicated temperatures for indicated times. Slopes reflect temperature-dependence of D branch ratio. At lower temperature more A goes to C' and less to F, so the rate of G production (slope) is smaller. Unpublished data, in part. See, also, Figure 6, Milkman and Hille (1966).

response curves at a single temperature will measure what fraction of the D actually proceeded to E. Figure 8 shows such an experiment. In our theory the D branch ratios at 39.5°, 40.5°, and 41.5° are 27%, 40%, and 55%, respectively. The data show that the expected ratios of slopes 1:1.5:2 are obtained.

E was previously shown (Milkman, 1963) to be a state which was protectable only by long intervals at temperatures below 35.5°. This is the last manifestation of rapid temperature adaptation. The $E \rightarrow F$ reaction is revealed by the disappearance of this mode of protection. Very few data are available, but the points on Figure 9 suggest that we have chosen an approximately correct time course. The dashed curve in the figure shows the consequence of removing the reaction which protects E ($k_{ED} = 0$). The difference between this line and the points shows the contribution of E-protection. The solid line is the expectation from the model as it stands. We must now determine Q_{1EF} . As with Q_{1DE} for the D branch ratio, Q_{1EF} governs the temperature dependence of the E branch ratio. If we take Q_{1ED} , k_{ED} , and k_{EF} at their postulated values and try three values: 1.4, 1.5, and 1.7 for Q_{1EF} , we find that the E branch point ratios fall to 50% at 33.5°, 35.0°, and 36.3°, respectively. At present our choice is 1.5 simply to have E-protection below 35.5°.

G formation

After 25 minutes at 40.5° the only reaction still proceeding is the conversion of F to G. As has been illustrated (Milkman, 1963), this is the best time to

measure Q_{1FG} . The curves in Figure 10 show that 1.5 is a good temperature coefficient over a 6° range. It was this unequivocal demonstration of a Q_{1FG} of 1.5 which forced us to seek a further explanation of the 2.3 temperature coefficient of pure dosage-response curves (Fig. 3). A multiplicative factor was discovered in the effect of the D branch point, as we have proven by the experiment of Figure 8. In Figure 3 we saw that Q_{1FG} and the factor from the D branch point are sufficient to explain the temperature dependence of the data.

So far we have not discussed how we chose k_{FG} . At 40.5° the estimated half-time of this reaction is 70 minutes. None of our experiments was long enough to reach the half-time of the F to G reaction at any temperature. Recall that at times much shorter than the half-time, an experimental curve is nearly linear so that all our predictions are based on a nearly linear production of G once all the reactions leading to F have ceased. Suppose we were to decrease k_{FG} by a factor of 100: the predictions of the model are unchanged. This surprising result is understood when we remember that all G-production curves will be reduced 100-fold

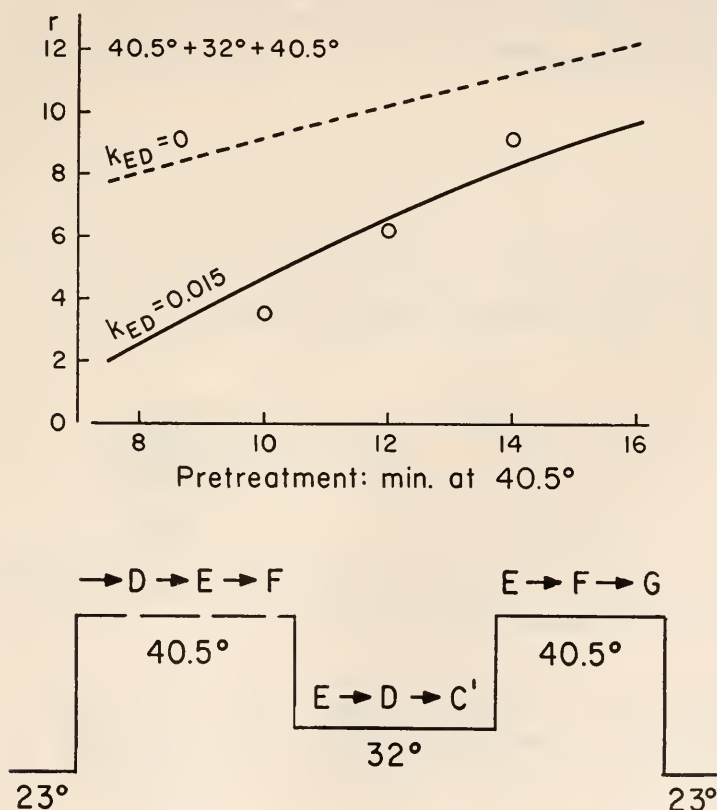


FIGURE 9. Response to 25 minutes at 40.5° after stated pretreatment and 1 hour at 32° . Rapid rise reflects in part the disappearance of E, whose protectability depends on the reversal of the $D \rightarrow E$ reaction. Were this reaction irreversible, the dashed line would represent the prediction.

and hence the values of G selected to correspond to posterior crossvein ratings from 0 to 10 will also be reduced. For this reason any choice of k_{FG} smaller than the present one will give equally satisfactory predictions. If we increased k_{FG} 3-fold, the 40.5° half-time would be reduced to 23 minutes, and all the dosage-response productions of Figure 1 would curve noticeably. As the agreement would no longer be satisfactory, we cannot increase k_{FG} . Thus our selection of 0.01 for k_{FG} is the maximum permissible. The arbitrariness of this choice could be eliminated if

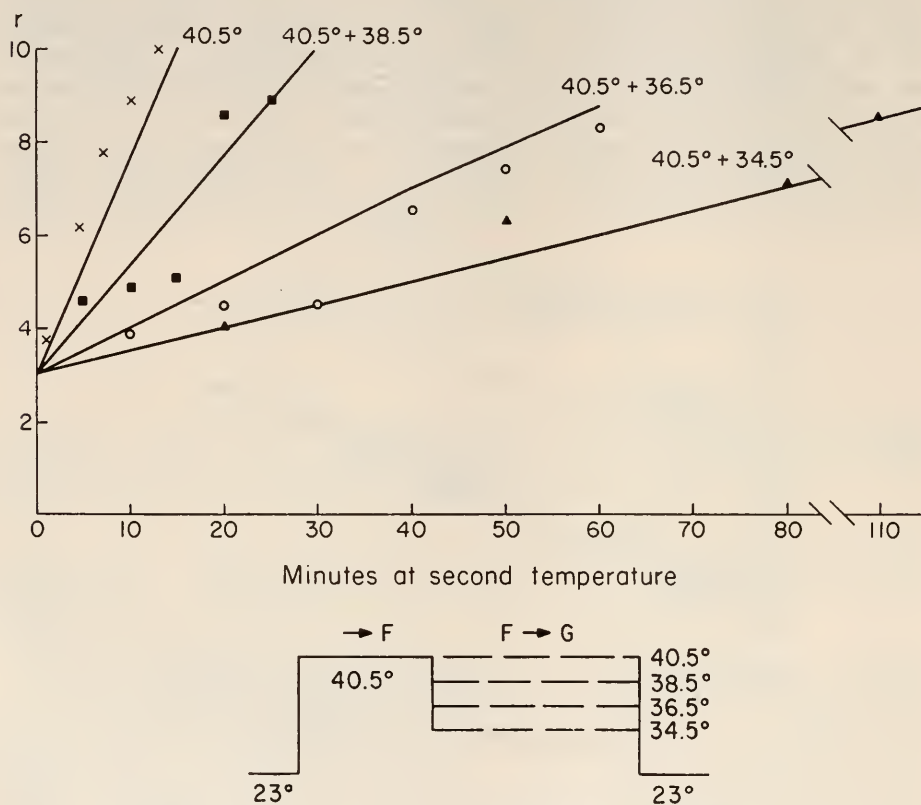


FIGURE 10. Response to various temperatures after exposure to 40.5° for 25 minutes. At this time, sole reaction is $F \rightarrow G$. Slopes reflect temperature coefficient of this reaction. Data from Table IV of Milkman (1963).

we could find a way of measuring the dosage-response behavior after treatments long enough to produce a bend in the curve. In experiments using protection to permit extending the exposure to 40.5° (Fig. 6) the treatment has not been long enough yet to show marked deviation from our nearly straight prediction.

The temperature-insensitive reactions

The five reactions with a Q_1 of 1.0 are primarily concerned with the different kinds of temperature adaptation. Their rates can be determined from the length

of the low temperature interval required to achieve a certain amount of protection. As the interval temperature from low temperatures up to 34° does not affect the rate of protection we have chosen Q_1 's of 1.0. Experiments depending on some of these relations have been published (Milkman, 1963, Tables VIII and IX, Figure 1). A more thorough demonstration appears in another paper (Milkman and Hille, 1966). In new experiments protection of E is found to be more rapid in the second 10 minutes of interval than in the first. It is because of this lag in E protection that we have chosen to let E revert to D before reaching a protected state, rather than having E form a protected state directly, as has been suggested before (Milkman, 1963).

DISCUSSION

We shall reconsider the four postulates of the model. The existence of the required protein A of postulate 1 is known only through defects produced after heat shocks. It would be desirable to demonstrate A in some other manner. Most meaningful of all would be the chemical isolation of a substance with the properties of A. Less direct and somewhat in the spirit of this investigation would be the analysis of genetic factors or chemical treatments which produce effects interacting with heat shock effects. So far none of these lines has been pursued successfully.

The kinetic sequence of postulate 2 is a very complex explanation for responses of pupae to heat shocks. Nevertheless, we are certain that we would fail to describe all the data if any single arrow were eliminated, regardless of how the other constants were changed. It is possible that some entirely different network of reactions could be as satisfactory as ours, but we do not believe that it could be simpler. One essential feature would be to have a branching scheme in order to get rapid temperature adaptation by a protected state. Possibly some reactions could be of a different order from first, but this is beyond the range of our computer to test.

The kinetic scheme of postulate 2 is also a complex description of the heat denaturation of a protein in a living organism. The conclusion that A is a protein rests on the now firm establishment of reactions with Q_1 's of 1.5 and 1.8, corresponding to activation energies of about 75 and 110 kcal. per mole. Steps in other protein denaturations have been shown to become rapid at temperatures from 40° to 60°, to have activation energies from 35 to 200 kcal. per mole to be reversible, to have temperature-insensitive reverse reactions, and to have branching mechanisms (Chase, 1950; Johnson, Eyring and Polissar, 1954; Kunitz, 1948).

The generality of temperature adaptation and heat prostration in plants and animals (Alexandrov, 1964; Precht, Christophersen and Hensel, 1955; Prosser and Brown, 1961; Ushakov, 1964) suggests to us that branching mechanisms of protein structural change might be a general explanation. Especially striking are the temperature adaptation and heat immobilization of streaming in epidermal cells of many plants (Alexandrov, 1964). These phenomena require practically the same temperatures and durations as those used here and might well yield to a similar analysis.

Postulate 3 is striking because it assumes that many tertiary structure states of a protein have full biological activity. In the experiments discussed, only four states

remained in significant quantity by the 26th hour of pupal development: A, C', F, and G. In the present scheme D could never remain longer than 10 minutes and E never longer than 60 minutes after treatment, each going to C'. B and C could be maintained in high equilibrium concentration by keeping the pupae at the temperatures around 30°. Undoubtedly the shift of the A-B-C equilibrium has an important role in making pupae whose entire pupal life was spent at a higher temperature more resistant to temperature effects. At any rate we have shown that A, C' and F are active states in posterior crossvein development.

The relationship between crossvein formation and G production shows the extreme sensitivity of this developmental process to changes in the amount of active protein. Defects follow on a 3% (or less, depending on the choice of k_{FG}) loss of active substance. This is not to say that A is a factor required only in posterior crossvein development. It might well be common and essential to many other processes whose sensitivity to small concentration changes is negligible. Indeed, it seems to be essential for life in that flies never live to achieve expected average ratings of 11 and 12 (about 9 G units). Other causes of heat death are also operating, so that in some kinds of heat experiments viability was zero even with very little G production. We should say also that other causes of posterior crossvein disturbance are also operating because in very long treatments at 36° to 38°, defects are produced when there has been almost no G production (Milkman, 1961, 1962). These phenomena remain unexplained. We find it remarkable that the wide range of temperature effects treated here can be described by the fate of a single substance so that the existence of other processes comes as no surprise.

The last postulate, saying that the parameters of the model are functions of the age, sex, genome, etc., has been adequately documented (Milkman, 1961, 1962). It opens the road to studying the development of protein A, its denaturation properties, and its translation into posterior crossvein. Hopefully by the time these properties are known we will also know protein A's chemical and developmental function.

The invaluable instructions on computer technique of Dr. Frederick Dodge and Dr. Charles Stevens; the facilities provided by Mr. John Hervey; the technical assistance of Mary Ann Cady and Tonja Knapp; and the clerical assistance of Maren Brown are gratefully acknowledged.

Part of this work was done at the Marine Biological Laboratory, Woods Hole. This work was supported by National Science Foundation Grant G-24023 to R. M.

SUMMARY

1. A complex array of high temperature effects on *Drosophila melanogaster* pupae is described in terms of a quantitative hypothesis. A branched series of reactions, first order in the reactant, provides a unifying basis for a set of adaptational, morphogenetic, and lethal effects.

2. The temperature coefficients of some of the reactions suggest that they may be specific, serial tertiary structure changes in an otherwise undescribed protein.

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THE EFFECTS OF HYPOPHYSECTOMY AND BOVINE PROLACTIN ON SALT FLUXES IN FRESH-WATER-ADAPTED *FUNDULUS HETEROCLITUS*

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Some of the characteristics of salt balance in the euryhaline killifish *Fundulus heteroclitus* have been outlined by us elsewhere (Potts and Evans, 1966). One of the major features of osmotic regulation in this fish is a marked reduction in the sodium and chloride fluxes on adaptation to fresh water. A similar reduction has been observed in several euryhaline teleosts including the stickleback *Gasterosteus aculeatus* (Mullins, 1950), the rainbow trout *Salmo gairdneri* (Gordon, 1962), *Fundulus kansae* (Fleming and Kamemoto, 1963), a blenny *Blennius pholis* (House, 1963) and the flounder *Platichthys flesus* (Motais and Maetz, 1964). Measurements of the drinking rate in *Fundulus heteroclitus* (Potts and Evans, 1966) and in *Platichthys flesus* (Motais and Maetz, 1964) show that the larger part of the influx in sea water takes place through the body surface, not through the gut. The site of this influx is uncertain but may well be the gills. Whatever the site it is clear that the body surface of sea-water-adapted fish is relatively permeable to ions but the low fluxes found in fresh-water-adapted fish show that they are relatively impermeable to ions.

Burden (1956) first demonstrated that hypophysectomized killifish were unable to survive in fresh water although they could survive in saline solutions. Later Pickford and Phillips (1959) showed that ovine prolactin promoted survival of hypophysectomized killifish in fresh water. Similar results have been obtained with several other genera including *Mollicnesia* (Mollies) (Ball, 1962), *Pocilia* (Ball and Olivereau, 1964), *Xiphophorus* (platyfish and sword-tails) (Schreibman and Kallman, 1962) and *Tilapia* (Handin, Nandi and Bern, 1964). In addition Schreibman and Kallman (1962) and Ball *et al.* (1965) have demonstrated that pituitary transplants enable hypophysectomized fish to survive indefinitely in fresh water. Schreibman and Kallman (1966) have recently reviewed the problem of hypophysectomy and survival in fresh-water fishes.

In the light of this evidence of the influence of prolactin on the osmoregulatory ability of teleosts the effects of hypophysectomy and prolactin on the salt fluxes of *Fundulus* have been examined.

MATERIALS AND METHODS

The fish used were *Fundulus heteroclitus*, weighing between 2 and 5 gm., collected by the Supply Department of the Woods Hole Marine Biological Laboratory. Sea-water-adapted fish were kept in running sea water which varied slightly

in salinity during the course of the work, from between 420 to 435 mM Na/L., 30.7–31.8‰ salinity, and between 19° and 21° C. in temperature. Fish adapted to 40% sea water, approximately isosmotic with the blood, and to fresh water were kept in aerated plastic tanks which stood in trays of running sea water so that the temperature of the tanks was approximately that of the sea water. Artificial fresh water was prepared by diluting sea water to 1 mM Na/L. with distilled water. The fish were fed every two or three days with chopped clam.

Sodium fluxes were measured by the methods described elsewhere (Potts and Evans, 1966). The fish were loaded by placing them in a solution containing ^{24}Na for two hours. The fish were then washed and the active solution replaced by an inactive solution. The efflux was measured after two hours in the inactive solution. All handling was kept to a minimum. The influx is obtained in absolute terms, *i.e.*, the amount of sodium which has entered the fish/hr., but the efflux is obtained in terms of the rate constant, *i.e.*, that fraction of the activity which leaves/unit time.

Hypophysectomy was performed by a method similar to that of Abramowitz (1937) and Handin *et al.* (1964). The fish were anaesthetized by 1:5000 MS 222. The parasphenoid was cut by iridectomy scissors and the pituitary removed by gentle suction. The animals were allowed to recover in 40% sea water and were used for experiment after normal feeding had been resumed, usually after one to two weeks. Bovine prolactin (NIH-P-BI, 13 International units/mg.) was provided by the NIH Pituitary Hormone Distribution Program. Prolactin was injected intraperitoneally in isotonic NaCl containing 168 mM NaCl/L., and 500 mg. prolactin/L.

Unless otherwise stated fish were injected with 20 γ prolactin/fish (*ca.* 5 γ /gm. fish) 48 hours before the experiment and injected again with 20 γ /fish 24 hours before the experiment. After the second injection the fish were transferred to fresh water. Each injection was equal to about 1% of the weight of the fish. This is equivalent to only half an hour of normal drinking in 40% sea water (Potts and Evans, 1966) or to one hour of urine production in fresh water (Stanley and Fleming, 1964). The injections are unlikely to alter water balance significantly 24 hours later.

RESULTS

After recovery from hypophysectomy the fish survived for at least several weeks in 40% sea water but after transfer to fresh water the fish became aesthenic in a few hours and the majority died within 24 hours. Burden found that hypophysectomized *Fundulus* would survive for several days in fresh water at 15° C. The more rapid deterioration of our fish may have been due to the higher temperature. Control fish survived indefinitely in the fresh water.

Sodium and bromide fluxes in hypophysectomized Fundulus

The rate constants of sodium and bromide efflux from hypophysectomized *Fundulus* in the two hours following transfer from 40% sea water to fresh water are shown in Table I. Salt loss is clearly much higher in hypophysectomized fish.

The rate of influx of sodium into hypophysectomized fish in fresh water was similar to that into normal fish. The mean rate of influx into a small series of

four hypophysectomized fish in the two hours following transfer from 40% sea water was $0.50 \mu\text{M}/\text{gm.}/\text{hr.}$ (range $0.63\text{--}0.31$). In normal fish fully adapted to fresh water the influx averages $0.58 \pm 0.10 \mu\text{M}/\text{gm.}/\text{hr.}$ ($N = 16$) (Potts and Evans, 1966). It is clear that the hypophysectomized fish is at a disadvantage in fresh water. As the sodium content of the fish will decline during the experiment the rate of loss will vary with time. However, if the initial sodium content of the fish were $60 \mu\text{M}$ Na/gm. (Potts and Evans, 1966) and the efflux rate constant were invariable, the initial rate of loss would be about $12 \mu\text{M}/\text{gm.}/\text{hr.}$ while the influx would be only $0.5\text{--}0.6 \mu\text{M}$ Na/gm./hr.

In normal fish adapted to fresh water the greater part of the sodium loss takes place through the body surface (*ca.* $0.36 \mu\text{M}/\text{gm.}/\text{hr.}$) while the remainder (*ca.* $0.22 \mu\text{M}/\text{gm.}/\text{hr.}$) is lost through the kidney and gut (Potts and Evans, 1966; Meier

TABLE I

Rate of constants of sodium and bromide efflux from normal and hypophysectomized Fundulus in various media; h^{-1} 20°C.

	Sea water	40% sea water	Fresh water (during 2 hours following transfer from 40% sea water)	Fresh water (adapted fish)
Sodium				
Hypophysectomized fish	0.446 ± 0.041 (7)	0.209 ± 0.07 (4)	0.192 ± 0.080 (11)	—
Normal fish	0.462 ± 0.024 (19)	0.175 ± 0.027 (14)	0.050 ± 0.013 (6)	0.0114 ± 0.003 (8)
Bromide				
Hypophysectomized fish	—	—	0.255 ± 0.032 (17)	—
Normal fish	0.492 ± 0.027 (9)	0.087 ± 0.007 (27)	0.134 ± 0.023 (18)	0.037 ± 0.004 (14)
			Mean $\pm$ S.E.	(No. of detcs.)

and Fleming, 1962; Stanley and Fleming, 1965). In an attempt to isolate the site of the increased loss of salt, hypophysectomized fish were transferred to fresh water four hours after the anus and excretory opening had been ligated. The mean rate constant of the sodium efflux during the two-hour period after transfer was then 0.208 ± 0.039 ($N = 9$). Hence the greater part of the loss probably takes place through the body surface.

The effect of prolactin

Hypophysectomized fish which received 20γ of prolactin every two days survived well in fresh water. The mortality that occurred, *ca.* 10%/week, may be attributed to the handling associated with the injections. The mean rate constant of 6 fish maintained in fresh water for 24 hours after receiving the second 20γ injection of prolactin was 0.026 ± 0.006 . Three hypophysectomized controls which had received no prolactin were all dead after 24 hours. In comparison normal fish, fully adapted to fresh water, have an efflux constant of 0.0114 ± 0.003 ($N = 8$) (Potts and Evans, 1966).

Normal fish adapted to fresh water contain about $50 \mu\text{M}$ Na/gm. If the hypophysectomized fish receiving prolactin contained the same quantity an efflux rate constant of 0.026 h^{-1} would correspond to a sodium loss of $1.3 \mu\text{M/gm./hr.}$ The mean measured influx into the four hypophysectomized fishes receiving prolactin was $1.0 \mu\text{M Na/gm./hr.}$ As the fish survived almost indefinitely, a balance must be struck between efflux and influx. How this is achieved requires further investigation. If the efflux is initially greater than the influx the blood concentration will decline. This may stimulate the uptake system above its normal levels. Alternatively as the total body sodium declines the absolute loss will decline in proportion even if the rate constant does not change.

DISCUSSION

The ability of prolactin to prolong the survival of hypophysectomized fish in fresh water has been known for seven years but the physiological basis of this action has not been clear. *A priori* two simple explanations present themselves. Prolactin might facilitate salt uptake in fresh water and/or it might reduce salt loss. In the latter case the loss might be reduced either at the body surface and/or in the kidney. The experiments reported above show that prolactin acts primarily in reducing salt loss. In the absence of prolactin the blood concentration will fall as loss exceeds uptake. Pickford, Pang and Sawyer (1966) have shown that when hypophysectomized *Fundulus heteroclitus* fails in fresh water the blood concentration lies in the region of $0.25\text{--}0.29 \text{ M/L.}$ compared with a normal concentration of 0.37 M/L. The effect of hypophysectomy on salt uptake requires further investigation. The survival of hypophysectomized fish for more than a few hours suggests that some compensation for the large losses has taken place. Any decline in blood concentration might be expected to stimulate salt uptake to some extent, but any such effect will be secondary.

The ligation experiments suggest that the greater part of the salt loss for hypophysectomized fish occurs at the body surface. The difference observed between ligated and non-ligated hypophysectomized fish, 0.208 ± 0.039 and 0.235 ± 0.089 , respectively, is not significant. Stanley and Fleming (1960) have shown that prolactin does affect renal function. In the plains killifish, *F. kansae*, prolactin was found to increase urine flow but to reduce urine sodium concentration in fish adapted to fresh water. It is not clear whether or not the overall renal sodium loss was increased or decreased.

The relationship between the effects of prolactin in these experiments and the normal function of the pituitary in the control of osmoregulation must now be considered. It has been shown that adaptation to fresh water by euryhaline teleosts is associated with a marked reduction in the extrarenal loss of sodium (Motaïs and Maetz, 1964; Potts and Evans, 1966). This is brought about in *Fundulus* by a reduction of the permeability of the body surface to ions (Potts and Evans) although in other species exchange diffusion plays an important part in the apparent reduction of sodium loss (Maetz, personal communication). In *Fundulus* a significant reduction in permeability may be induced by the immersion of sea-water-adapted fish in fresh water for only 10 minutes. Once induced the reduced permeability is maintained for several hours. It is tempting to suggest that this reduction of permeability is brought about by the release of a prolactin-like hormone from the

fish pituitary. It is significant in this respect that hypophysectomized fish survived well in sea water and the rate constants of sodium exchange were similar to those in normal fish (Table I). Similarly the rate constant of hypophysectomized fish in fresh water, and hence their permeability, was similar to that of fish adapted to 40% sea water.

A prolactin-like hormone has been identified in *Fundulus* and in the fresh-water carp but not in the marine cod (*Cadus*) or hake (*Urophycis*) (Grant and Pickford, 1959). This teleost prolactin-like hormone resembles mammalian prolactin but is not identical with it. The teleost prolactin-like hormone possesses red eft water drive activity (Grant and Pickford, 1959) but lacks pigeon crop sac stimulating activity (Nicoll and Bern, 1964). Similarly frog prolactin shows some but not all the characteristics of mammalian prolactin (Chadwick, 1966).

A prolactin-like hormone is not essential for the survival of all teleosts in fresh water. Both the eel *Anguilla anguilla* and the golfish *Carassius auratus* will survive in fresh water following hypophysectomy (Fontaine, Callamand and Olivereau, 1949; Schreibman and Kallman, 1966), although the eel at least also produces a prolactin-like hormone (Ball and Olivereau, 1964). In the case of the eel survival may be due to its very low permeability. Normal silver eels will survive "readily" in glass-distilled water for three months and the rate of sodium loss in fresh water is of the order of $11 \mu\text{M}/\text{kg}/\text{hr.}$ compared with $580 \mu\text{M}/\text{kg}/\text{hr.}$ in *Fundulus* (Chester Jones, Henderson and Butler, 1965). At low temperatures even hypophysectomized *Fundulus* will survive in fresh water for several days (Burden, 1956) so it must be very close to salt balance in these conditions.

Attempts by the authors to induce a state of low permeability by the injection of prolactin into sea-water-adapted *Fundulus*, comparable with that induced by a short treatment with fresh water, were not successful. This may indicate that further stimuli are required to bring about a state of low permeability in addition to prolactin alone. On the other hand more success might be obtained with ovine prolactin or better still with a fish prolactin-like hormone. Ovine prolactin supports hypophysectomized *Fundulus* rather better than bovine (Pickford, Robertson and Sawyer, 1965).

Pickford, Pang and Sawyer (1966) have recently suggested that prolactin prolongs survival in fresh water by its action on the fish mucous cells. The greater part of the extrarenal sodium exchange probably takes place through the gills rather than through the general body surface. Mucus might reduce loss over the gills by increasing the thickness of the non-stirred layer but a direct action on the gill epithelium is also possible.

Olivereau (1966) found that prolactin had a thyroid-stimulating action in hypophysectomized eels (*Anguilla*) but thyrotropin does not prolong survival in hypophysectomized platyfish (*Xiphophorus maculatus*) in fresh water (Schreibman and Kallman, 1966).

There is some evidence that hypophysectomy reduces urine flow in fresh water while prolactin increases it again. Chester Jones *et al.* (1965) found that urine flow in hypophysectomized eels was less than half normal while Stanley and Fleming (1965) found that hypophysectomized *F. kansae* had low rates of urine production which were increased by prolactin. Their results may imply that prolactin increases water permeability but a low permeability to water would not be a disadvantage in fresh water.

This work was supported by N.I.H. grant no. GM 1030-03. We are also indebted to the N.I.H. Endocrinology Study Section for the supply of the prolactin.

SUMMARY

Hypophysectomized *Fundulus* in fresh water lost sodium several times as rapidly as normal fish. The greater part of the loss takes place extrarenally. Prolactin reduces the loss almost to normal levels, thus prolonging survival. The possible functions of prolactin in osmoregulation in normal fish are discussed.

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THE MECHANISM OF BURROWING IN THE POLYCHAETE WORM, *ARENICOLA MARINA* (L.)

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New techniques of recording pressure changes and activity have increased our knowledge of the fluid dynamics of burrowing in *Arenicola marina* (L.) (Trueman, 1966a). The new information, together with an understanding of the habits of the lugworm, derived from the extensive researches of Wells (1961), allow an assessment of the mechanism of burrowing to be made.

Initial entry of a worm into sand is brought about by the eversion of the proboscis at comparatively low coelomic pressures (2–6 cm. water pressure) and when several segments have passed beneath the surface a series of pressure peaks commence, each of about two seconds duration. They occur at intervals of 5–7 seconds, and as burrowing progresses increase in amplitude up to 110 cm. During burrowing, waves of peristaltic contraction pass along the trunk from the posterior segments to the anterior buried region of the worm where they appear to develop into high pressure peaks by the synchronous contraction of the longitudinal muscles of all or part of the trunk segments. The fluid of the essentially single trunk coelom acts in a hydraulic system which allows the force produced by the longitudinal muscles of the posterior trunk to be transferred to the anterior end, there to be utilized in burrowing. The principal function of the high pressure is to anchor the anterior end during the contraction of the longitudinal muscles. Each time the pressure increases the anterior end is pressed firmly against the substrate, while the posterior trunk is pulled into the burrow.

The purpose of this article is to consider further observations of the movements made by *Arenicola* during burrowing and to compare the mechanism with that of other animals, in particular bivalve molluscs.

MATERIAL AND METHODS

Observations were made of the burrowing of *Arenicola* of 15–20 cm. length both at Hull and at the Marine Biological Laboratory, Millport, using specimens which would burrow rapidly. Direct visual observation of burrowing into sand could be made from above or from the side, through the glass of an aquarium tank. The latter was largely, but not always, unrewarding as even when the worm was close to the glass a thin layer of sand could obstruct detailed observation. Accordingly a technique (Trueman, 1966a) of continuously recording the pressure imparted to the sand by a burrowing worm was further developed by use of a more sensitive pressure transducer (Statham, Model P 23 BB, maximum sensitivity 0.4 cm. pressure/cm. pen deflexion) which was coupled to a multichannel pen recorder. Both instruments were obtained from E. & M. Instrument Company Inc.

Worms were allowed to burrow over a glass tube (3 mm. bore) buried in the sand with its external opening covered by a coarse nylon mesh to prevent entry of sand grains. This was connected by pressure tubing to the transducer, and pressure applied to the adjacent sand either by a plunger or by an *Arenicola* burrowing caused a negative response. The explanation of this may lie in the dilatant properties of the sand (Chapman and Newell, 1947), for the applied pressure disturbs the packing of the sand-water system and tends to cause water to be drawn in. In a full account of this technique (Hoggarth and Trueman, 1966) it is emphasized that all recordings must be interpreted by direct visual observations but that with this proviso it serves as a useful method of determining the activity of an animal, invisible beneath the sand yet without any obstruction by electrodes. Although burrowing was recorded in this manner for about 50 worms, direct observations of burrowing movements were only satisfactorily made on 5 occasions when the events were marked on the recording by means of a manually operated key. Coelomic pressures during burrowing were recorded as previously with a Bourdon transducer obtained from the E. & M. Instrument Company Inc. (Trueman, 1966a).

EXPERIMENTAL RESULTS

The recording of external pressures derived from an *Arenicola* burrowing in sand covered by several cm. of water consists of a series of negative pressures whose amplitude varies with the distance of the worm from the recording device. These pressures were observed to correspond to the swelling of the anterior segments and to a marked increase in the turgidity of the entire trunk region (Fig. 1a).

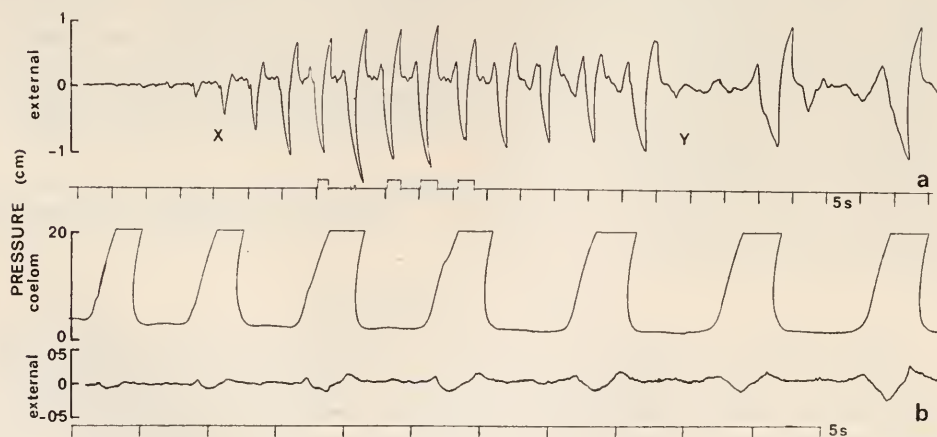


FIGURE 1. Recordings of the pressures produced by *Arenicola* in sand (external) and in the coelom during burrowing. a, sequence from the commencement of burrowing (extreme left) showing gradual increase in amplitude of the negative swings as penetration proceeds (at X) and the reduction of their frequency (at Y) after 6 branchial segments have passed into the sand. Visual observations of the swelling and turgidity of the anterior segments are indicated above the time trace. b, simultaneous recordings of coelomic and external pressures, commencing with four anterior segments beneath the sand. Lower amplitude of the external pressures due to greater distance from the recording device; flat tops of the coelomic pressure trace caused by saturation of the recorder.

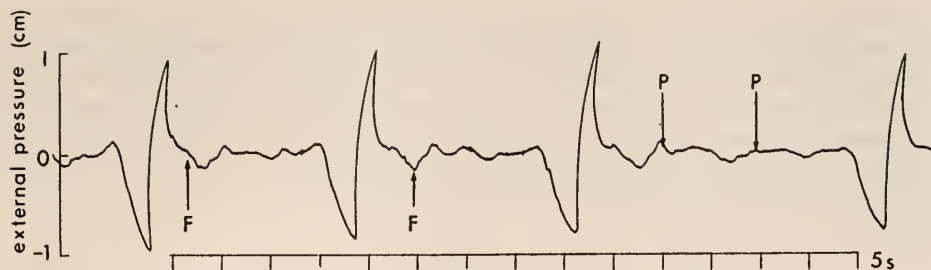


FIGURE 2. Recording of the pressures produced in sand by *Arenicola* when burrowing against glass with the trunk almost completely beneath the surface. Flanging (F) and proboscis eversion (P) were marked by direct observation of the anterior segments.

Synchronous recordings demonstrate that the negative pressures correspond to the pressure peaks in the coelom (Fig. 1b). At the commencement of burrowing the latter increase in amplitude with penetration (Trueman, 1966a; Fig. 6) and a similar feature occurs in respect of the negative pressures (Fig. 1a, X). This indicates that until a firm anchorage is obtained, maximum pressures are not exerted on the substrate.

The external pressure recorded fluctuates continuously between the negative peaks and it has not been possible to interpret these changes in detail. The most marked positive peak, following immediately after the negative pressure, may be due to the dilatant properties of the sand-water system. Proboscis eversion and the occurrence of flanging were marked by visual observations, through the side of a glass tank. Flanging (Fig. 3b) was most clearly observed on the first three trunk segments where the fleshy parapodial ridges each form an annulus which may be raised suddenly into a sharply projecting flange (Wells, 1944, 1961). Flanging and proboscis eversion were never observed at peak pressures but occurred between these (Fig. 2). Conversely the anterior 4 or 5 segments became very dilated when the maximum pressures were recorded (Fig. 3a). High pressures were clearly not synchronized with proboscis extrusion nor was there any apparent forward movement of the head of the worm at this phase of digging activity. It was previously (Trueman, 1966a) considered likely that the high coelomic pressure contributed to forward movement of the head of the worm but in the light of the present observations this appears to be incorrect.

After *Arenicola* has burrowed for 1 to 1½ minutes the frequency of the negative pressures often shows a marked reduction (Fig. 1a, Y). When recording the coelomic pressure a similar feature was observed and was interpreted as being due to the lack of development of the full muscular power of the worm, possibly because of fatigue (Trueman, 1966a). Waves of peristaltic contraction pass forward along the trunk during burrowing and develop into pressure peaks upon reaching the anterior segments. As burrowing proceeds each peristaltic wave does not produce high coelomic pressures or dilation and accordingly the negative pressures also occur less frequently. Proboscis extrusion and flanging continue, however, throughout the interval between maximum pressures. The proboscis effects the initial entry of the worm into the sand by a scraping action (Wells, 1961) and very likely continues to scrape away the substrate when more deeply burrowed. Re-

peated proboscis extrusion between pressure peaks is thus probably related to the progression of the worm. The resistance of the substrate to penetration increases with depth of burial (Trueman, Brand and Davis, 1966b) and the longer interval between pressure peaks allows time for more extrusions of the proboscis and extension of the head. During studies of burrowing by bivalves similar observations have been made, indicating that the amount of probing by the foot increases with depth of burial (Trueman, Brand and Davis, 1966a).

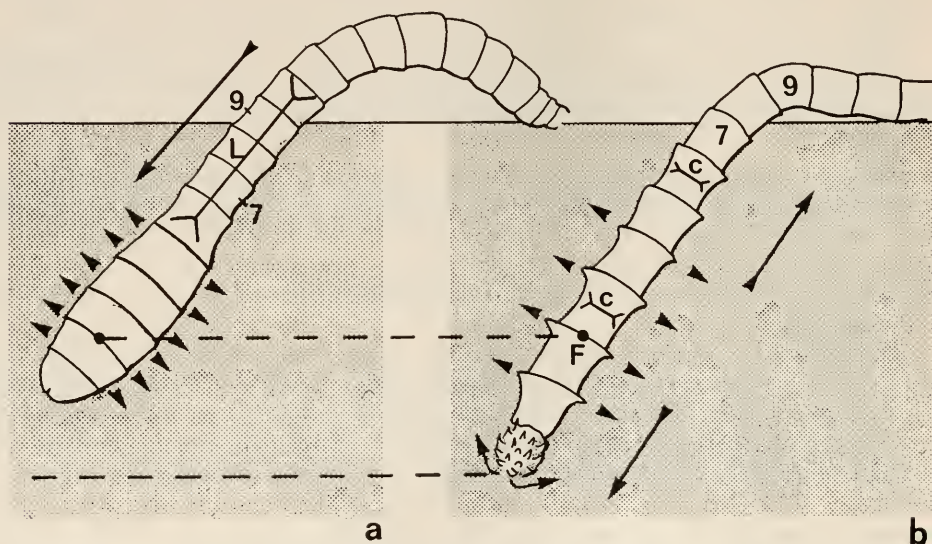


FIGURE 3. Diagram of two successive stages of burrowing of *Arenicola*. a, shows the anterior segments dilated to form an anchor (arrowheads), allowing the worm to penetrate the burrow (solid, tailed arrow) on contraction of the longitudinal muscles (double arrow, L). b, shows the flange anchor (arrowheads) and eversion of the proboscis (solid arrow). b, immediately follows a, at the fall of coelomic pressure, and involves elongation by the contraction of circular muscles (double arrow, C). Provided the flanges afford an anchorage, e.g., at F, on the second chaetigerous annulus, the head pushes forward and the posterior trunk segments retract from the burrow (solid, tailed arrows). Movement between a and b is also indicated by broken lines drawn between comparable parts of the worm and by the numbering of the segments.

BURROWING ACTIVITY OF *ARENICOLA*

On the basis of recordings (Figs. 1 and 2) and direct observations it has been determined that two principal conditions of the anterior trunk segments occur successively during burrowing activity. These are first, dilation, caused by the high coelomic pressures and secondly, flanging, accompanied by proboscis eversion (Fig. 3). Both of these conditions involve the anchorage of some part of the anterior region of the worm and in a normal burrowing sequence the type of anchorage alternates between the dilation anchor (Fig. 3a) and the flanging anchor (Fig. 3b).

The peristaltic wave passing forwards along the trunk forces coelomic fluid into the head with the contraction of the circular muscles posterior to segment 7 (Fig.

3a). This causes some increase in coelomic pressure, partial dilation of the most anterior segments (Trueman, 1966a) and is immediately followed by the contraction of the longitudinal muscles of the trunk. This brings about maximum dilation and a firm anchorage of the head, so allowing the posterior of the trunk to be pulled forward into the burrow. The effect of the head segments exerting pressure on a dilatant substrate is to make the sand-water mixture more resistant so that the chaetae can grip and the body-wall adhere to form a firm anchor. This stage of burrowing is equivalent to that described by Wells (1961) as an "anti-seagull" reflex, which he demonstrated by allowing a worm to burrow down the stem of a large glass filter funnel, the stem being closed by rubber tubing and a clamp. When the *Arenicola* was halfway into the stem, pulling the hinder end backwards caused the dilation of the anterior segments and resulted in a tenacious grip. This experiment has been repeated with a pressure transducer attached to the coelom and gave rise to high internal pressures which persisted while the hind end was being pulled.

In normal burrowing the high coelomic pressure and the dilation anchor are sustained by the contraction of the longitudinal muscles for not more than 2 seconds, being followed by the relaxation of these muscles and the contraction of the circular fibers so causing elongation of the worm. This elongation appears on the surface of the sand as a re-emergence of the posterior trunk segments from the burrow as the pressure drops (Trueman, 1966a). Contraction of the circular muscles completely eliminates the dilation anchor but this is replaced by the flanging anchor (Fig. 3b). This anchorage allows the second or third chaetigerous segment to remain static at elongation of the worm so that the segments behind will be pushed backwards from the burrow and those in front forwards into the substrate as the proboscis everts. This condition is shown diagrammatically in Figure 3b where the second annulus is arbitrarily taken as a fixed point about which movement backwards and forwards occurs. Wells (1954), in a detailed account of the mechanism of proboscis movement, considered that the head of *Arenicola* narrows and lengthens during the first stage of proboscis extrusion. This is in accord with the observation that eversion takes place when the dilation anchor is lost as the worm elongates.

The burrowing activity of *Arenicola* consists of the following stages: (1) Probing forward by the head, proboscis eversion obtaining initial penetration into the sand with no large pressures recorded. (2) Several segments buried, allowing a dilation anchor to form (Fig. 3a) with accompanying high pressures and the pulling forward of the worm into the burrow. (3) Relaxation of longitudinal and contraction of circular muscles, resulting in the lengthening of the worm and the production of the flange anchor (Fig. 3b). Further penetration of the substrate is then obtained by proboscis extrusion. (4) The second and third stages are repeated cyclically until burial is complete.

High coelomic pressures correspond to stage (2) but as the worm elongates during the third stage, the pressure drops sharply to the equivalent of little more than 2 cm. of water. The function of the high pressure is both to obtain an anchorage and to compact the sides of the burrow. External pressure recordings of *Arenicola* in normal U-shaped burrows show occasional strongly negative pressures, comparable to those recorded during digging, which suggest that high

coelomic pressures are used in the natural habitat, possibly to consolidate the burrow wall. Somewhat similar observations have been made in respect of burrowing in the earthworm (Roots and Phillips, 1960).

DISCUSSION

The digging activity of bivalve molluscs that burrow into soft substrates, such as *Cardium*, *Donax*, *Anodonta* or *Ensis* (Trueman, 1966b; Trueman *et al.*, 1966a), makes an interesting comparison with that of *Arenicola*, for all are well adapted for

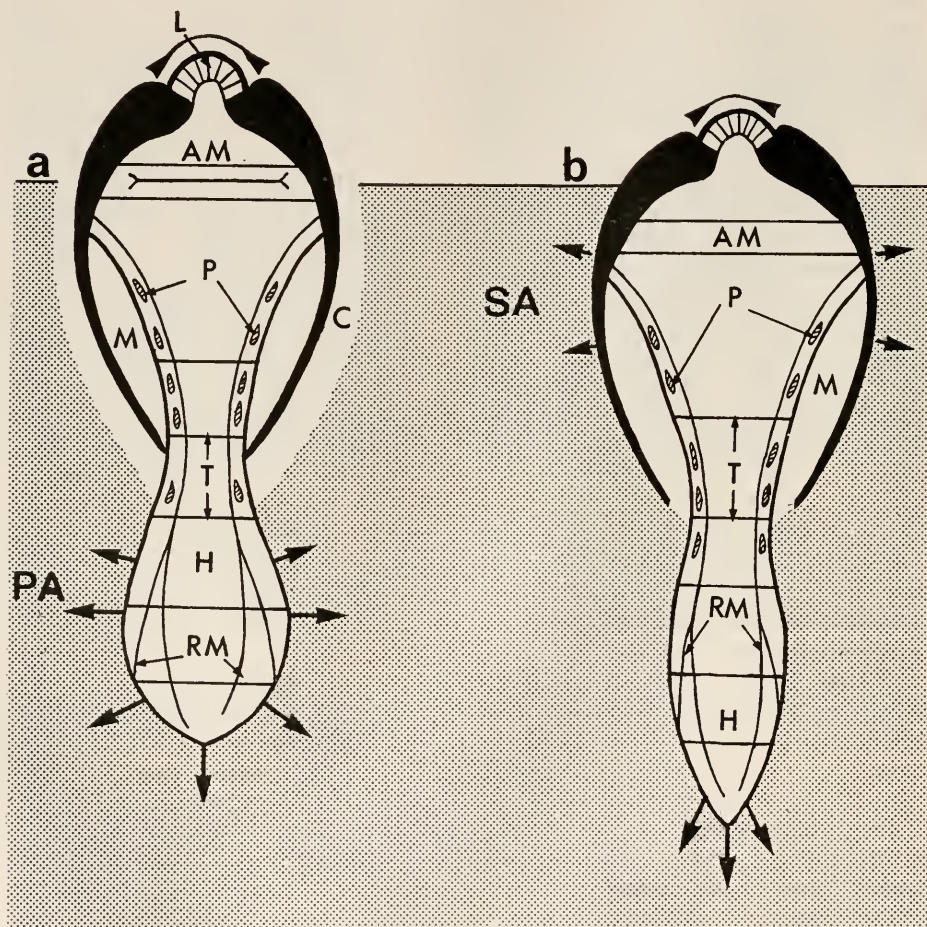


FIGURE 4. Diagram of two successive stages of burrowing of a generalized bivalve mollusc showing pedal (PA) and shell (SA) anchorages (arrows). a, valves adducted (double arrow, AM) producing pedal dilation and a cavity (C) in the sand around the valves. Contraction of the retractor muscles (RM) then causes the shell to be pulled down. b, valves reopened and pressed against the sand by the elasticity of the ligament (solid, double arrow, L) holding the shell fast when contraction of the protractor (P) and transverse muscles (T) causes pedal protraction. AM, adductor muscle; H, pedal haemocoel; M, mantle cavity.

burrowing. The latter has an essentially single coelomic system in contrast to the double system of the bivalves, which consists of the haemocoel, the hydrodynamic equivalent of the coelom in *Arenicola*, and the pallial system. Many bivalves thus have the advantage of being able to eject water from their mantle cavity during digging to loosen the sand adjacent to the shell (Fig. 4a, C). In both the thrust used in initial penetration is limited by the weight of the animal since any force in excess of the weight causes the animal to be pushed back from the sand. Further penetration of a bivalve consists of a series of step-like movements, each termed a "digging cycle," which were well recorded by Quayle (1949) in a study of the digging movements of *Venerupis*. Each digging cycle involves the integration of adduction and the reopening of the valves with retraction and protraction of the foot. Adduction causes high pressure in the haemocoel and as a consequence the foot becomes swollen to form a pedal anchor (Fig. 4a) (Trueman, 1966b). Immediately after adduction the retractor muscles, equivalent to the longitudinal muscles of *Arenicola*, contract, pulling the shell down and sustaining

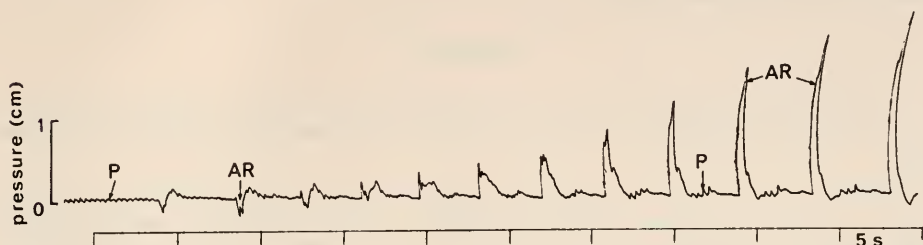


FIGURE 5. Recording of the pressures produced in sand by the burrowing of *Macra subtruncata*. At the commencement the bivalve is lying on the sand and the foot penetrates by probing (P). A succession of adductions of the valves and pedal retractions (AR) follow, giving first (left) negative swings, when the foot alone is beneath the sand, to be replaced by large positive pressures (right) when the valves have entered the sand. These are caused by ejection of water from the mantle cavity at adduction.

the pedal pressure and anchorage. Subsequently the opening moment of the hinge ligament opens the valves and presses them against the substrate (Fig. 4b) so forming a shell or secondary anchor. This holds the animal firmly whilst the foot extends by contraction of the protractor and transverse pedal muscles, which in this respect are the equivalent of the circular muscles of the body wall of *Arenicola*. The pedal and shell anchors of a bivalve correspond to the dilation and flange anchors of *Arenicola*, respectively.

The entire pattern of burrowing activity of a bivalve as recorded by the external pressures (Fig. 5) is very similar to that of the lugworm. It consists of a series of digging cycles each of which produces high pressure at adduction-retraction (AR). These are seen as negative pressures at the commencement of burrowing (Fig. 5, left) as in recording of *Arenicola*, but as depth of penetration increases (to the right, Fig. 5) and the shell enters the sand, water is ejected from the mantle cavity into the substrate and causes the succession of positive pressures (Hoggarth and Trueman, 1966). Between the "AR" peaks, when the shell anchor is applied, numerous probes (P) are made by the foot. These correspond to the pushing forward of the head of a worm from the flange anchor which occurs between each

negative pressure recorded. Although the structures used in digging in *Arenicola* and in bivalves are anatomically quite different there is a fundamental similarity in the mechanism that they employ.

Whilst discussing the burrowing of worms Clark (1964) considered that the method of burrowing used by all soft-bodied animals is essentially the same. He suggested that part of the body wall is first dilated to form an anchor while the head is forced into the substrate by contraction of the circular muscles, and that secondly the anterior end of the worm dilates to form a new anchor while the body is drawn downwards by contraction of the longitudinal muscles. These two anchorages correspond respectively to the flanging and dilation anchors of *Arenicola* or to the shell and pedal anchors of a bivalve. Essentially the same mechanism is used by other soft-bodied animals to burrow into sand, e.g., *Nephtys* (Clark and Clark, 1960), *Urechis* (Fisher and MacGinitie, 1928). Detailed knowledge, derived from continuous recordings of activity and internal pressures, is so far limited to *Arenicola* and members of the Bivalvia. It is hoped to extend these observations in the near future.

SUMMARY

1. The burrowing activity of *Arenicola* has been studied by means of direct observations and recordings of pressure changes both internally and in the adjacent sand.

2. Maximum coelomic pressures correspond to the swelling of the anterior segments to form an anchor (dilation anchor) which allows the posterior trunk segments to be pulled into the sand and the sides of the burrow to be compacted. This condition occurs alternately with the occurrence of flanges on the anterior segments as the worm elongates by contraction of the circular muscles.

3. The flanges tend to form an anchor (flange anchor) from part of the anterior region so that lengthening forces the head into the substrate, as the proboscis everts. At the same time the posterior trunk region undergoes some retraction from the burrow.

4. Essentially the same method is used by all soft-bodied animals to dig into sand, notably in bivalve molluscs. In this group a pedal anchor is formed by the foot becoming swollen by the hydrostatic pressure derived from adduction of the valves immediately before the shell is pulled down by the pedal retractor muscles. The shell is subsequently held still by the opening of the valves against the substrate (shell anchor) while the foot is protracted by the intrinsic pedal musculature.

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ABSTRACTS OF PAPERS PRESENTED AT
THE MARINE BIOLOGICAL LABORATORY

1966

ABSTRACTS OF SEMINAR PAPERS

JULY 26, 1966

Effects of ultraviolet radiation with special reference to racial differences in coloration. GEORGE SZABÓ.

Single and repeated exposures of ultraviolet radiation of erythema doses were delivered on human subjects of Caucasian (redhead and Mediterranean), Negro and Mongoloid races, to compare the response of exposed areas (forearm) with that of the unexposed regions (hip or abdomen) of the same individual.

There was a marked difference in response between various races and between different regions of the integument of the same individual. The same erythema dose provoked no visible reddening in the unexposed (hip) areas of Negroes or Mongoloids, whereas visible erythema developed in redheaded Caucasians. There was no visible erythema in Caucasians or in other races when the forearm was exposed to ultraviolet. The melanocyte population did not show a clear tendency toward increase after single exposure, but in the case of the hip, three subjects out of 6 showed an increase 10 days after radiation. In the case of the forearm, after fluctuation at 5th day post-radiation, there was no such increase in melanocyte frequency. After multiple doses of radiation, there was an increase in melanocyte frequency both in the forearm and on the abdomen. In the forearm, this increase was much smaller than in the abdomen. After the cessation of multiple radiation, the elevated melanocyte population showed a tendency to revert to its original lower density. Electron microscopical studies have revealed that non-irradiated melanocytes contain mostly pre-melanosomes, although the neighboring Malpighian cells may be full of melanized melanosomes. After radiation, however, the melanocytes are full of melanized melanosomes, and the number of melanosomes inside Malpighian cells also increases.

The work was supported by grants CA 05401-04-06, N.I.H., and USPHS Career Development Award, K3-GM-14,987.

Microtubules and morphogenesis. The role of microtubules in the development of the primary mesenchyme in the sea urchin embryo. LEWIS G. TILNEY AND JOHN R. GIBBINS.

In sea urchin embryogenesis the complex and species-specific skeleton of the pluteus larva is produced exclusively in the cells of the primary mesenchyme. During their development the cells of the primary mesenchyme undergo a predetermined sequence of changes in shape which result finally in an oriented syncytium. It is in this syncytium that the skeleton is deposited. At each stage examined cytoplasmic microtubules are disposed in patterns which correlate with the shape of the cell at that stage. The shape of the cells during their development then may be controlled by the microtubules and therefore the microtubules could be responsible ultimately for the orientation and configuration of the skeleton. To test this hypothesis developing embryos were exposed to two types of agents known to affect the microtubules of the mitotic spindle as well as cytoplasmic microtubules. Agents of a type which produce tubule breakdown, colchicine and hydrostatic pressure, inhibited the development

of the primary mesenchyme and resulted in the loss of microtubules. The cells tended to spherulate and no skeleton was produced. D_2O , an agent which "freezes" or stabilizes microtubules, also stopped development but the microtubules remained and the cells retained their asymmetric form. It would appear, then, that the sequential disassembly and reassembly of cytoplasmic microtubules into new patterns is essential for the form differentiation of the cells of the primary mesenchyme. Disruption of normal development results from interference with this system or with its control.

Research supported by USPHS Grant #2G-707-05 to Dr. Keith R. Porter.

Cilia regeneration in the sea urchin embryo. WALTER AUCLAIR AND BARRY W. SIEGEL.

Late gastrulae of *Paracentrotus lividus* regenerated cilia following deciliation in hypertonic sea water. The rate of growth, following a ten-minute lag period, was $1 \mu/5 \text{ min.}$ for approximately two hours. The rate then slowed down for the next 2–3 hours until the cilia reached a final length of 24–25 microns, equal to that of the original cilia. The apical tuft cilia did not regenerate to their original length of 70–75 microns.

Neither dactinomycin (25 and 50 $\mu\text{g./ml.}$) nor puromycin (50 and 200 $\mu\text{g./ml.}$) affected the rate of growth of the regenerating cilia. Dactinomycin (25 $\mu\text{g./ml.}$) also did not affect incorporation of C^{14} -l-leucine and C^{14} -l-glutamic acid (each at concentrations of 0.1 $\mu\text{c./ml.}$) into the proteins of regenerated cilia during the regeneration phase, but total embryo protein incorporation was reduced. Puromycin (50 $\mu\text{g./ml.}$) inhibited both total embryonic and ciliary protein synthesis drastically.

The data indicate that ciliary protein synthesis is continuous and under the control of a pre-existing, long-lived messenger RNA template. In addition, the data suggest that there is present an intracellular pool of pre-formed ciliary proteins that aggregate into cilia even when protein synthesis is stopped.

Supported by Office of Scientific Research, Office of Aerospace Research, U. S. Air Force, Grant no. 964-66.

AUGUST 9, 1966

The effect of 15° C. on the stages of normal development of Fundulus heteroclitus. D. R. SHANKLIN.

Selection of morphological stages for a developmental series is partially arbitrary, although some phases of embryogenesis warrant separate designations. Further, functional integration and morphogenetic movements require a reasonable synchrony which is a prerequisite for comparative staging. The Armstrong-Child series for *Fundulus* (*Biol. Bull.*, 128: 143–168, 1965) qualifies as a base sufficiently detailed for quantitative study. Their series was constructed at 20° C. In early June, 1966, the running sea water at the M.B.L., Woods Hole, was 15° C. Seven batches were fertilized and followed at 15° C. Two were returned to 20° C. at gastrulation (stages 12 & 15) and two at onset of circulation (stage 25); all were followed until hatching (stage 34). Ordinary plots of stages *versus* total time were hyperbolic; semi-logarithmic plots were biphasic linear, with a steeper slope after about 12 hours. The 15° curve lay to the right on the time plot and on the rectangular plot gradually moved further to the right. The changes in the intervals between stages were examined. This revealed a great variability in the ratio of interval times (15°/20°) which ranged from 1.04 to 3.0 up to stage 33; the interval ratio 33–34 exceeded 4.72. When the interval ratios were plotted against time, with stages marked in, a fluctuant curve resulted which alternates great delay with little delay. This suggests a critical effect of cooling at certain points, which, when relieved metabolically or morphokinetically, affects stage achievement only slightly until the next critical period is reached. The phases of great delay were: mid-cleavage, preblastula cell multiplication, gastrulation, growth and organodifferentiation prior to general circulation, and the acquisition of axial linearity and motile strength preceding hatching.

This work was supported by The John A. Hartford Foundation, New York, New York.

Rate of hatching of Fundulus heteroclitus at 20° C. and the effect of prior exposure at 15° C. D. R. SHANKLIN.

Hatching of *Fundulus* results from two phenomena related insofar as is known only temporally: release of an enzyme from the oropharynx and strong muscular action of the tail. With temperature maintained at 15° C., Armstrong-Child stage 34 is reached in about 400 hours as compared to 228 hours at 20° C. (*Biol. Bull.*, 128: 143-168, 1965). Even though a full range of axial movement occurs and both the mouth and gill slits are open and show a gentle rhythmic action, no hatching or activities seen immediately prior thereto (*e.g.*, shrinkage of yolk sac) were observed even up to 136 additional hours at stage 34, over 5 times the interval 33-34 usually required at 20° C. This great delay was previously noted by Gabriel (*J. Exp. Zool.*, 95: 105-147, 1944). Certain writings imply that hatching is a reasonably simultaneous process, interrupting only transiently the course of development. A small group of eggs maintained at 20° C. took 191 hours for hatching to reach 100%. Two groups of 108 and 112 eggs, respectively, required 164 hours (these were at 15° C. until stage 25) and when the per cent hatched is plotted against time an S-shaped curve results with good linearity between 30% and 90%. Several groups were put at 20° C. after various preselected stages were reached. These also showed a significant spread of total hatching time (0-100%) at stage 34. The plot of total exposure time at 15° C. against rate of hatching yields a rough but reasonable direct relationship whose approximate average slope is $\log 1/t = 0.74 + 0.00125d$, where t = time in hours to reach 100% hatching once it has begun and d = duration of exposure at 15° beginning at fertilization. The study does not show which hatching action is affected by the treatment or whether both are.

This work was supported by The John A. Hartford Foundation, New York, New York.

Fine structure of tight junctions. JEAN-PAUL REVEL AND MORRIS J. KARNOVSKY.

After fixation in the presence of neutralized lanthanum nitrate, we have observed that the intercellular space becomes filled with an intensely electron-opaque material. Presumably a form of lanthanum hydroxide acts as tracer and penetrates in the tissues *via* these spaces. In the mouse, the most intensively studied species, we have successfully delineated the extracellular space in heart, liver, kidney, intestinal epithelia, smooth muscle, transitional epithelium and the intermediate line of nerve myelin. The lanthanum compound also stains the intermediate line of tight junctions and we describe here in particular the results obtained in the intercalated disc of the mouse heart and in the liver. The stained intermediate line is wider than in unstained preparations, but the total width of the tight junctions is unchanged. Seen in oblique view the tight junctions show a series of striations with a periodicity of 90 Å, while in face-on view one observes a hexagonal pattern with a center-to-center distance of 90 Å. The appearance of the pattern does not change as the temperature is varied from 0° C. to 37° C. The patterns observed are identical to those reported by Robertson at the electrical synapse of the Mauthner cell in the goldfish brain, but the use of lanthanum allows one to view the hexagonal pattern with much better contrast. Similar patterns have also been reported in negatively stained, isolated liver cell membranes by Emmelot and Benedetti. Species variations, and the physiological implications of the hexagonal pattern, were discussed.

Supported by grants HE 09125 and GM 11380 from the NIH, USPHS. MJK was the recipient of a Lederle Medical Faculty Award, and JPR of a Research Career Development Award.

Fine-structural basis for chemical and electrotonic transmission in a parasympathetic ganglion. A. J. DARIN DE LORENZO AND GERALD R. BARNETT.

The ciliary ganglion of the newly-hatched and adult chicken contains synapses of a unique type. Preganglionic nerve fibers enter the ganglion and terminate in calyciform or cup-like endings upon postganglionic nerve cells. Since the postsynaptic cells have no dendrites the endings are entirely axo-somatic and they embrace as much as 65% of the cell surface. Electrophysiological studies of these synapses have demonstrated both chemical and electrical transmission.

Examination of these junctions with the electron microscope has revealed the following organization. Postsynaptic cells are surrounded with a myelin sheath which covers the synapses and the perikaryon except at the axon hillock region. The synaptic membranes of the calyx are separated from the postsynaptic cell by a cleft about 250–300 Å wide. Structural specializations seen in chemical synapses consisting of accumulations of synaptic vesicles, thickened membranes and clusters of mitochondria are seen throughout the calyx. Only in the area of the axon hillock do the synaptic membranes exhibit tight junctions characteristic of electrotonic transmission sites seen elsewhere. At these sites synaptic vesicles are conspicuously absent. The synaptic cleft is obliterated by a fusion of the inner components of the unit membrane comprising the synaptotlemma. Cross-striations are often resolved in the fusion sites. Of particular interest is the occurrence of structural characteristics implicating both chemical and electrotonic transmission in the same synaptic membrane separated by distances measuring less than a micron. Hexagonal structures seen in other tight junctions have not as yet been demonstrated.

Supported by USPHS Grant NB 02173.

The fine structure of vesicles associated with excitatory and inhibitory junctions.

G. D. PAPPAS AND M. V. L. BENNETT.

Our previous studies on spinal motoneurons which innervate the swimbladder muscle of *Opsanus tau* indicate that excitatory transmission to these cells is electrotonic and mediated at axo-somatic junctions where the apposing membranes are fused. Synchronous firing of the motoneurons is probably aided by dendro-somatic junctions where the apposing membranes are also fused. On the other hand, typical axo-somatic synapses where a synaptic gap of about 200 Å occurs provide a morphological basis for hyperpolarizing inhibitory postsynaptic potentials which must be chemically mediated (*Ann. New York Acad. Sci.*, 137: 495, 1966). In both classes of axo-somatic junction, the axon terminals contain similar vesicles, although clustering of vesicles to the pre-junctional membrane is more common in junctions where there is no membrane fusion.

Uchizono (*Nature*, 207: 642, 1965) and Bodian (*Science*, 151: 1093, 1966) suggest that in electron micrographs of formalin- or glutaraldehyde-fixed tissue inhibitory synapses are characterized by synaptic vesicles with ellipsoid profiles. In contrast, the vesicles in excitatory synapses appear uniformly round. After glutaraldehyde fixation, two distinct classes of vesicles cannot be differentiated in the toadfish swimbladder nucleus. Axon terminals showing regions of fusion contain a spectrum of vesicular profiles. Terminals where an extra-cellular space is maintained also contain round to ellipsoid vesicles. In addition, both kinds of terminals contain tubular elements more frequently than after fixation in osmic acid.

AUGUST 16, 1966

Chemical studies of directin. ANDREW F. HEGYELI.

A biodynamic agent was found in extracts from human urine, which is identified by its induction of directional growth of malignant and not of normal cells *in vitro*, and was therefore tentatively named directin (D).

The chemical separation, purification, properties, and possible biological significance of D were discussed. The properties of D were presented under the following headings: stability, enzyme degradation studies, molecular weight, group reagent reactions, chemical analyses, and chromatographic studies. Evidence was presented that D has a molecular weight around 600 and is a hydrolyzable phosphate derivative, possibly containing a sugar moiety. The loss of directional growth activity is related to the splitting off of one or more phosphates.

The effect of D on D-treated rats carrying Walker 256 tumors can only be explained by postulating that D is involved in the energy transformation processes in the cell. When unstained fixed histological sections of the tumors were excited by fluorescent light at 450 μmicrons, the tumor cell nuclei emit a rapidly quenched light.

ADP, ATP, and inorganic phosphate are known to be important in energy transformations in the cell. ATP and ADP have about the same molecular weight as D, and contain hydrolyzable phosphate groups. They were therefore investigated in the same biological assay used for

D. (This was done at the suggestion of R. Poirier.) Both ATP and ADP induced directional growth of KB cells *in vitro*. AMP and inorganic pyrophosphate were inactive, while orthophosphate gave borderline activity. Evidence was presented that D is not identical with either ATP or ADP. It has a different solubility pattern and 100 times less N content for the same biological activity. The negative test with AMP suggests that the active site, responsible for the directional growth activity in both ATP, ADP, and D, is an easily hydrolyzable phosphate group.

This research was supported by Battelle Memorial Institute.

In vitro and in vivo studies of directin. RUTH JOHNSON-HEGYELI AND ANDREW HEGYELI.

A biodynamic substance was discovered last year in extracts from human urine and tentatively named directin (D) because of its induction of directional growth of malignant cells and not of normal cells *in vitro*. Chemical evidence obtained to date indicates that D is a hydrolyzable phosphate derivative, possibly a sugar of about 600 molecular weight.

This paper reported on further biological studies of D. These were illustrated by slides of cell cultures and histological sections, followed by a brief movie of D-treated KB cells.

The action of D was considered under three headings: cells, medium, and substrate. Its action on the following types of cells was discussed: solid tumor cell lines, virus leukemia strain, normal cell lines, normal cell strains, and primary cultures from 14-day- and 7-day-old chick embryos. Directin has three microscopically detectable effects in malignant tissue cultures: (1) It is growth-retarding, (2) it changes the morphology of the cells, and (3) it changes the growth pattern of the cells. After 16 to 20 hours' exposure to D the cells become bipolar, there is increased communication, and formation of tight junctions between neighboring cells, and polarization of growth. Immediately following mitosis the nuclei move to opposite poles of the two cells and the nucleoli are also lined up in a row at this time. The D effect with cell cultures grown in different media and on conducting metal surfaces rather than on insulating glass was discussed.

Experiments with D-treated rats carrying Walker 256 solid tumors showed that D has three effects on tumor cells *in vivo*: (1) It changes the morphology of the cells, particularly the nuclei, (2) it induces areas of parallel orientation of tumor cells, and (3) the nuclei of treated unstained tumor cells emit rapidly quenched light when excited by light at 450 μ microns in fluorescent microscope.

This research was supported by Battelle Memorial Institute.

The effects of some inhibitors on the temperature-dependent component of the lobster axon resting potential. JOSEPH P. SENFT.

The resting membrane potential of the lobster axon increases 5–8 mV when the temperature of the perfusion solution is increased 10° C. This potential change is about twice that predicted if the axon membrane potential followed that expected for a potassium ion electrode potential. When the inhibitors 2,4-dinitrophenol, cyanide, and azide were added separately to the axon perfusion medium the potential change was reduced to about 1.4 times that predicted for a potassium ion electrode potential. Assays of axons exposed to these inhibitors showed that ATP levels were reduced to about one-fourth that obtained for control axons. Ouabain added to the perfusion medium reduced the potential change to that expected for a potassium ion electrode potential. These results suggest that the lobster axon resting potential changes with temperature as a result of the activity of an electrogenic ion pump.

Supported in part by NSF grant GB-332.

Studies on a major protein from isolated sea urchin egg cortex. R. E. STEPHENS AND R. E. KANE.

Isolated cortex was prepared by the method of Sakai, involving lysis of eggs of the sea urchins *Colobocentrotus atratus*, *Tripleneustes gratillia*, and *Arbacia punctulata* in 0.1 M

MgCl₂, followed by light homogenization and differential centrifugation to remove cytoplasmic contaminants. Analytical ultracentrifugation revealed that distilled water extracts of the cortex were composed of only a few components, the most prominent of which was a hypersharp 7S material, identified as a calcium-precipitable protein originally described by Kane and Hersh as a component of whole egg extracts. Essentially all of the 7S protein recovered from whole egg lysates could be obtained from isolated cortex alone. The protein constituted 2-4% of the total cell protein and 50-95% of the soluble cortical extracts, depending upon the species.

The protein was only sparingly soluble in solutions of ionic strength greater than 0.2, producing a particle with a sedimentation constant in the range of 10-12 Svedbergs. The Yphantis short column sedimentation equilibrium method indicated that the 7S protein from *C. atratus* had an average molecular weight of 725,000 at pH 7.5, 386,000 at pH 11.5, 277,000 in 8 M urea, and 299,000 in 8 M urea plus 2% mercaptoethanol; strong association was evident in all cases and, hence, these values should be considered tentative. The pH and urea subunits were reversible to the original protein by dialysis into neutral buffer.

The amino acid composition showed no significant species variation and was characterized by high glutamic acid, valine, and proline contents. Optical rotatory dispersion measurements indicated virtually no α -helix to be present, a finding consistent with the proline content.

Divalent cation-precipitated fibers of the 7S protein were highly birefringent but lengthened and lost birefringence upon treatment with 0.001 M EDTA; addition of divalent cations caused the fibers to shorten and regain birefringence.

Supported by Public Health Service Grant GM 14363 from the Division of General Medical Sciences.

GENERAL SCIENTIFIC MEETINGS

AUGUST 22-25, 1966

Abstracts in this section are arranged alphabetically by authors. Author and subject references will be found also in the regular volume index, appearing in the December issue.

Squid lens development in compounds that affect microtubules. JOHN M. ARNOLD.

The squid lens develops by fusion of elongate cellular processes (lentigenic processes) which grow out of a specialized group of cells (lentigenic cells) in the front of the developing optic vesicle. These processes wrap about each other to form an "onion bulb-like" structure (lens primordium) in which elaboration of an electron-dense lens material takes place. The lens primordium increases in size by continued application of lentigenic processes from the lentigenic cells. Eventually the elaboration of this dense lens material obliterates the cytoplasm of the lentigenic processes. During the phase of active outgrowth the lentigenic processes contain many microtubules, Golgi-derived vesicles, mitochondria, and ribosomes. Fixation with glutaraldehyde and post-osmication demonstrates electron-dense areas (probably a precipitation product) in the lentigenic cells during the time of lens material elaboration but not in other tissues of the eye.

Treatment of the stage 25 embryo (Arnold, 1965) with 10^{-3} M or 10^{-4} M colchicine for 24 hours causes the microtubules to disappear and the lentigenic cells to undergo a very dense accumulation of ribosomes and an increase in size of the electron-dense areas. Washing the treated embryos for an additional 24 hours in normal sea water causes a reversal to the typical appearance and a reappearance of the microtubules. Colchicine treatment of the stage 22 embryos prevents outgrowth of the lens primordium. Treatment of the stage 22 or stage 24 embryo with 0.1 M mercaptoethanol apparently allows the lentigenic processes to continue their outgrowth but they no longer fuse to form a lens primordium. Mercaptoethanol also causes the microtubules to become solid, irregular, and uniformly electron-dense.

It is therefore indicated that microtubules may play a role in transport of materials to the developing primordium. However, their specific tubular structure is not essential in this transportive role.

Aided by a grant from the National Science Foundation, GB-3202.

Ribonucleic acid in membranes of developing cells. ARYA K. BAL, PAUL L. KRUPA AND GILLES H. COUSINEAU.

Previous work on RNase-treated membrane systems in *Allium cepa* L. root-meristem cells showed removal of a ribonuclease-sensitive material from unit membranes. In the present studies possible contamination of ribonuclease with lipase and proteases (which might have led to similar findings in the initial investigation) was circumvented by the use of (1) boiled ribonuclease, or (2) lipase and trypsin.

The tissues were fixed in a mixture of 5% glutaraldehyde-paraformaldehyde at 23° C. After repeated washing, the tissues were incubated with each enzyme (dissolved in distilled water) at 37° C. for 6 to 18 hours (pH 6.5). The concentrations of lipase and trypsin were 1 mg./ml. Ribonuclease (0.5 mg./ml.) was boiled for 5 minutes and cooled to 37° C. before use. Controls were run concurrently in distilled water. The enzyme treatment was followed by post-osmication and embedding in Epon in the usual manner.

In root-meristem cells treated with lipase and trypsin there was no appreciable change in cell structure compared with the controls. On the other hand, boiled ribonuclease was effective in removal of electron-dense material between the leaflets of the lipoprotein membranes.

These results were confirmed in gastrula cells of *Arbacia punctulata*. After treatment with boiled ribonuclease, the bilaminar organization of the unit membranes became more evident, due to removal of the ribonuclease-labile component. This was seen in the nuclear envelope, endoplasmic reticulum, Golgi complex, plasma membrane, and in the membranes surrounding pigment granules.

Thus, under these experimental conditions, it is very likely that RNA is present (in association with, or as an integral part of) the membrane systems of the embryonic cells in the tissues studied.

This work was supported by grants-in-aid of research from the Damon Runyon Memorial Fund for Cancer Research (grant #DRG-918), the National Research Council of Canada (grant #731-741), and the Society of the Sigma Xi.

Investigations of the subunit structure of Limulus hemocyanin. FRANK C. BANCROFT, ROBERT C. TERWILLIGER AND K. E. VAN HOLDE.

Preliminary studies have involved the determination of conditions for obtaining the various subunits of hemocyanin, and the characterization of the subunits by sedimentation coefficient ($s^{0}_{20,w}$) and molecular weight. The Yphantis sedimentation equilibrium method was used for molecular weight determinations. Buffers were of ionic strength 0.1. Whole blood was allowed to clot; it was then centrifuged and filtered to yield stock solutions of hemocyanin. These stock solutions were of pH 7.3, and contained about 36 mg./ml. hemocyanin, mostly as a 62S component, with some 16S component also present. Dilution with pH 6.6 phosphate to 7.0-0.36 mg./ml. resulted in a progressive dissociation of the 62S component to a 40S component with dilution, suggesting a protein concentration-dependent equilibrium. However, passage through Sephadex G-25 equilibrated with pH 6.6 phosphate, or dialysis against this buffer, caused dissociation to 40S at 7 mg./ml., suggesting involvement of a dialyzable component of the blood. Dilution of the stock to 0.36 mg./ml. with either pH 6.6 phosphate + 0.01 M Mg^{++} (I), or pH 6.6 cacodylate $\pm$ 0.01 M Mg^{++} or Ca^{++} (II) yielded the 60S component. Incubation of these solutions for 24 hours at 25° C. caused dissociation in I, whereas dissociation was prevented in II. Dilution of the stock with pH 10.6 bicarbonate yielded a 5.2S component. Under various conditions a 16S component was observed. Dilution of the stock with pH 4.6 acetate yielded a 25S component, which dissociated largely to the 5.2S component after 24 hours at 25° C., or overnight dialysis vs. pH 4.6 acetate, $\pm$ 0.01 M Mg^{++} . Molecular weights of 6.0×10^4 and 1.94×10^6 were obtained for the 5.2S and 40S components, respectively, assuming partial specific volumes of 0.710 and 0.740, respectively. A tentative model for the subunit structure has been formulated. Electron micrographs by Van Bruggen suggest the 16S component is composed of eight 5.2S components. The relative sedimentation coefficients of the larger subunits suggest that the 25, 42, and 60S components are dimers of the 16, 25, and 42S components, respectively. The molecular weights obtained are consistent with this model.

This work was supported in part by NIH Grant #5 T1 GN25608.

The fine structure of the eye of the scallop, Pecten irradians. ALLEN L. BELL.

Available electrophysiological evidence indicates that the proximal retina of the scallop eye gives an "on" response to light while the distal retina gives an "off" response. It is not known whether the distal retina's response is a case of primary inhibition or is a result of inhibition by the proximal retina.

Preliminary studies of the fine structure of both retinas show processes close to the distal retinal cells which contain clear vesicles 600 to 700 Å in diameter. This suggests a mechanism for modification of the response of the distal retina by some other cell. Studies are in progress to determine the origin of these processes.

All of the surface epithelial cells of the scallop eye have microvilli at their surface. The surface epithelium proximal to the pigmented region of the eye is thrown into folds which bring the microvilli of opposing cells into a configuration resembling a rhabdomere. If these rhabdomere-like structures were photoreceptors, one would expect to find nerves or processes passing centrally from them. None have been seen up to this point in the investigation.

Other tissues of the eye which were examined include: the cornea, the pigmented surface epithelium, and the tapetum. A full description of these tissues will be given in future reports of this work.

Further observations on the thermodynamics of the living mitotic spindle. ROBERT M. CAROLAN, HIDEMI SATO AND SHINYA INOUÉ.

With the polarizing microscope the birefringence (measured as retardation) of the mitotic spindle of *Pectinaria gouldi* oocytes was measured at temperatures from 5° C. to 38° C. in both artificial H₂O sea water and artificial 42% D₂O sea water. Below 21° C. increased temperature enhanced spindle retardation; the plot, $\log B/(A_0 - B)$ versus $1/\text{temp. (}^\circ\text{K)}$ follows a van't Hoff relationship giving a straight line. B is spindle retardation and A_0 the asymptote B approaches. ΔH in H₂O was 82 ± 8.5 and in D₂O 59 ± 12 kcal. ΔS in H₂O was 286 ± 29 and in D₂O 208 ± 40 eu. Both differences are significant to the 0.001 level. The ratios of the H₂O and D₂O values are identical to those reported previously although the absolute values are greater, due presumably to technical improvements.

These data are consistent with the hypothesis that spindle retardation reflects the reversible association of protein "monomers" into linearly aggregated polymers. At 17° C. the equilibrium constants of the D₂O and H₂O reactions are equal, meaning the ratios of effective "monomer" concentration to effective polymer concentration are equal at this temperature. Since D₂O increases the spindle retardation and presumably the polymer concentration, this suggests D₂O increases the pool of "monomers" available for polymerization. In addition D₂O must have some direct effect on the polymerization reaction itself since it alters its thermodynamic parameters.

Above 21° C. increased temperature reduced spindle retardation. A van't Hoff relationship exists here also but with negative ΔH 's and ΔS 's. These values appear less negative in D₂O than H₂O.

After exposing the cells to temperatures as high as 36° C. normal bipolar spindles reappeared at room temperature. After exposure to 37° C. tripolar, tetrapolar and other anomalous spindles appeared. After exposure to 38° C. no spindles could be recovered. These effects are identical in both D₂O and H₂O even though in H₂O spindle birefringence becomes undetectable around 34° C. while in D₂O it persists to 37° C.

Aided by grants from the National Cancer Institute CA 10171 and the National Science Foundation, GB-5120.

Ultrastructural relationships between the developing oocyte and auxiliary cells in adult Artemia salina. REV. JOSEPH D. CASSIDY, O.P.

Fine-structure associations were surveyed in ovarian germ plasm, nurse cells and follicular-like epithelium of *Artemia salina*. Stock #3 females were cultured in filtered sea water supplemented with 50 g./liter NaCl. Dissected ovaries were fixed for 2 hours in 5% glutaraldehyde prepared in Millonig's buffer at pH 7.4 with 10% sucrose, stained with 1% OsO₄ and saturated

uranyl acetate, dehydrated in ethanol and embedded in Maraglas. Thin sections were examined in the Siemens Elmiskop IB. Adjacent $1\ \mu$ sections were taken for cytochemical reactions and studies with Zeiss phase contrast illumination. Differentiated regions of the trophocyte-oocyte complex were analyzed in longitudinal view.

Spherical nurse cell nuclei are highly polyploid with prominent nucleoli rich in RNA. The nuclear envelope is studded with closely packed annuli. Cytoplasmic profiles of numerous Golgi, ellipsoidal mitochondria and polysomes are interpreted as morphological concomitants of synthetic activity. A true syncytium is lacking, as evidenced by extensive membrane systems which separate nurse cell nuclei. The nurse cell-oocyte interface consists of a border of interdigitating microvillar projections. These cortices of nurse cells show a disorganized array of microtubular elements, free RNP particles, and scattered filaments of endoplasmic reticulum. Droplets, particles and intact mitochondria appear in transit *via* micropynocytotic vesicles extending from the oocyte border. At the cortex of the oocyte distinct types of yolk spheres and lamellae are observed in close contact. The germinal vesicle gives a lobate appearance in transverse and tangential view. A previously unreported cell type enveloped part of the oocyte boundary. It has a distinctively elongate nucleus, an irregular acellular outer wall and could occupy the position of a follicle cell if one is present. Along its attachment to the oocyte border are pale lipid vacuoles. Submorphology and distribution suggest a possible role in lipogenesis of the developing oocyte.

Induction of the shell gland by transplanted polar lobes in Ilyanassa. JAMES N. CATHER.

To determine the cell lineage of the shell gland in *Ilyanassa* the 2d and 2c micromeres were marked with carbon granules. The 2d micromere derivatives form the shell gland but 2c derivatives are later incorporated into the mantle. Deletion of 2d causes shell abnormalities, but in about 10% of the cases the shell appears normal but somewhat small. Deletion of 2c also causes characteristic shell abnormalities. After deletion of both 2d and 2c no shell forms. When 2c is marked and 2d deleted the shell gland is marked. Any $1/4$ embryo can form internal shell masses. In 1013 isolated ectoblasts (-3A, -3B, -3C, -3D) no shell material formed. Ectoblast with any single macromere formed shell with good morphogenesis, as did ectoblast plus 4d. Posterior ectoblast plus 4d, equivalent in size to isolated whole ectoblast, formed external shell. Properly oriented ectoblast grown in contact with a polar lobe formed shell in up to 70% of the cases; in one-half of these the shell was external. Deletion of the macromere from an ectoblast plus 3C series of embryos at intervals through the first day resulted in embryos which did not form shell. Ectoblast in contact with polar lobes for 6 days, then separated, developed shell on the seventh or eighth day. Thus: (1) Neither ectoderm nor endoderm in isolation will form shell but any ectoderm-endoderm combination has the histogenetic ability to form shell material; (2) the endoderm exerts its influence between the second and sixth days of development; (3) only those combinations including or in contact with polar lobe cytoplasm through the third quartet stage undergo normal morphogenesis; (4) transplanted polar lobes can induce ectoderm to form shell; (5) the histogenetic ability to form shell is suppressed or unactivated when polar lobe cytoplasm is present, except in the D quadrant.

This work was supported by NSF Grant GB-1035.

Respiration studies with the shark: biochemical aspects. C. LLOYD CLAFF, ARMAND A. CRESCENZI AND ARTHUR P. RICHMOND.

The shark lives in an environment of sea water with a pH of 8.0 to 8.6. Its gills are laved with this alkaline fluid. It is likely that he extracts oxygen from his environment more easily than we humans do from air. It is interesting to note, in passing, that we humans for the first nine months live in the uterine fluid of a pregnant woman with a pH of 7.9 to 8.0, the calculated pH of the Pre-Cambrian era sea water.

I suggested the use of the shark as an experimental animal in connection with our work on the Pulsatile Pressure Membrane Oxygenator for open-heart surgery. I did this because

the shark gets rid of some of its nitrogenous waste of metabolism through its gills as well as its cloaca. Therefore, its blood is always loaded with waste material.

Dr. Crescenzi and I spent four months in 1964 at the Lerner Marine Laboratory, Bimini, Bahamas, on this work. Our first attempts were failures, although we were using a cellophane membrane on the dialyzing side of our instrument and Teflon membrane on the oxygen gas side.

A native boy helping us suggested dialyzing with sea water, and our success was immediate. Dr. Crescenzi noticed that we were getting an alkaline shift in our blood passing through the oxygenator. It was the typical "Bohr" effect of the dissociation curve of blood oxygen to the right with increased CO_2 and alkaline pH of 7.5.

This was first pointed out by Niels Bohr over 50 years ago. Those of us who concerned ourselves with designing oxygenators had completely ignored this important phase of respiration.

The biochemistry of respiration is as important as the physical diffusion factors and must be incorporated into any artificial membrane device for maximum efficiency.

Basic research can and does provide answers for clinical medicine and public health.

Supported by the Single Cell Research Foundation, Inc., 5 Van Beal Road, Randolph, Massachusetts.

Cleavage and differentiation of the vegetal half of the Ilyanassa egg after removal of most of the yolk by centrifugal force. ANTHONY C. CLEMENT.

By holding *Ilyanassa* eggs "upside down" on the centrifuge in a gelatin-sea water gel, yolk was driven into the animal hemisphere; the lipid zone, clear zone, and pronuclei were forced into the vegetal hemisphere. Eggs showing this reversal of the usual pattern of stratification were freed from the gel and centrifuged in a raffinose solution until they separated into light and heavy halves. Nucleated light halves derived from the vegetal hemisphere, and free of most of the yolk normally contained in the vegetal hemisphere, formed a polar lobe and cleaved unequally in the normal pattern. Many such yolk-poor vegetal fragments differentiated lobe-dependent structural features (eyes, shell, foot, etc.), and some developed into small but well-proportioned veliger larvae. The morphogenetic influence of the polar lobe region is thus not displaced by centrifugal force of the strength employed in these experiments (2000 *g* for about six minutes), nor is it dependent upon the presence of the normal yolk complement.

This work was supported by NSF grant GB-1572.

Electron microscopy of the gas-secreting gland of Portuguese man-of-war. D. EUGENE COPELAND.

Portuguese man-of-war (*Physalia physalis*) secretes carbon monoxide as the gas to inflate the float or pneumatophore. An oval patch of epithelium (ectodermal in origin) at the base of the inner layer of the float is believed responsible for the gas production. Based on a study of animals collected off Gay Head (Martha's Vineyard, Mass.), it was previously reported that the gas-secreting epithelium possessed no mitochondria or recognizable Golgi material. Vesicles at the free surface could be the gas-release mechanism. An entirely different picture is now presented, based on *Physalia* collected in the Gulf of Mexico off the Mississippi Delta. It is now obvious that the gas-secreting epithelium is extremely sensitive to adverse environmental conditions (in this case, temperature). The gas-secreting epithelium contains large, closely packed mitochondria in the end of the cell toward the gas surface. The mitochondria have few, short cristae; a dense matrix occupies the internum of the mitochondrion. Well developed Golgi and a large population of lysosomes are found in the general area between the mitochondria and the nucleus. Mitochondria are seen incorporated into autophagic vacuoles and appear to then degenerate into lysosomes. The nuclei, previously reported as surrounded by a satellite of vesicles, are instead surrounded by extensive double membranes that enclose flattened cisternal spaces. The free surface of the cell is thrown into irregular projections of various lengths. There is no obvious sign of gas release by vesicles. The vesicles previously reported undoubtedly arise by collapse and reordering of the surface projections.

Support provided by National Science Foundation (GB-676) and USPHS, National Institutes of Health (GM 06836).

The contribution of the abdominal nerve cord to the chromatic physiology of the prawn, Palaemonetes vulgaris. ERNEST F. COUCH, MILTON FINGERMAN AND EDWARD W. STOOL.

The ratio of red pigment-dispersing hormone to concentrating hormone in the abdominal nerve cord of *Palaemonetes* is higher than for any other organ of this prawn. Experiments were designed to determine the role of this structure in the color change process as compared with the function of the neuroendocrine structures in the cephalothorax. When both eyestalks were removed from animals whose nerve cord was cut between the thorax and abdomen, they darkened as is typical of eyestalkless prawns but slower than animals with intact cords. Dark-adapted eyed prawns injected with an extract of tritocerebral commissure to induce blanching re-dark-adapted more slowly if their ventral nerve cord was cut. These findings are readily explained by assuming that the prawns with cut nerve cords had less darkening hormone available to them. However, eyed prawns with cut cords adapt to a black background at the normal rate, showing that sufficient hormone for this function to occur at the normal rate can be released from the cephalothorax. The nerve cord was divided into three portions and assayed on pale prawns to determine the linear distribution of red pigment-dispersing hormone. Cords from freshly collected animals and those maintained for six days with cut cords were compared. No increase in activity was found in the anterior end of the cord from the prawns kept six days. The lack of accumulation in the anterior part of the cord strongly suggests that hormone is not carried forward through the cord for release in the cephalothorax. Heat and electrical stimulation of the cord caused darkening in isolated abdomens, demonstrating thereby direct release of darkening hormone from the cord. Light microscopy revealed neurosecretory cells within the ganglia of the abdominal nerve cord which could be the source of chromatophorotropins.

Supported by Grant GB-5236 from the NSF.

The challenge of actinomycin D on early development of sea urchin embryos. GILLES H. COUSINEAU, PAUL L. KRUPA AND ARYA K. BAL.

Eggs of the sea urchin *Arbacia punctulata* were fertilized and allowed to develop at 23° C. with gentle agitation. Aliquots were then taken every 30 minutes and placed into flasks containing actinomycin D at a final concentration of 50 µg./ml. At 330' post-fertilization (p.f.) the embryos were pulsed for one hour with either uracil-2-C¹⁴ or DL-leucine-1-C¹⁴. Samples of unfertilized eggs with and without actinomycin, and fertilized eggs without and in continuous contact with the drug were also pulsed with the labeled precursors at that time. Embryos challenged with the antibiotic during development incorporate leucine-C¹⁴ into protein. Setting the incorporation in the 330-minute control at 100, relative incorporation rates for the actinomycin-treated embryos were computed as follows: 0', 59; 30', 55; 60', 60; 120', 63; 150', 65; 180', 78; 210', 77; 240', 88; 270', 98; 300', 99. A 40% decrease in incorporation into protein of C¹⁴-leucine was also observed between 6 and 7 hours p.f. in bulk labeling experiments, although the actinomycin embryos in earlier stages had consistently greater activity. Incorporation of uracil-C¹⁴ into RNA gave the following results: 0', 10; 30', 21; 60', 29; 90', 30; 120', 33; 150', 60; 180', 62; 210', 65; 240', 79; 270', 80; 300', 82; control 330', 100. Embryos challenged during the first two hours of development continued to divide but failed to differentiate, while those treated between 150 and 210 minutes p.f. showed increasing orderly differentiation; nevertheless formation of normal blastulae was only observed with those embryos challenged later than 210 minutes p.f. The consistently greater RNA synthesis obtained from all challenged embryos, and certainly the increase observed in the 30- and 60-minute samples, would indicate that once initiation of DNA-dependent RNA synthesis has occurred, such activity is in some way resistant to actinomycin D, even when given in massive doses over long periods. Further work is in progress to characterize the RNA produced.

This work was supported by grants-in-aid of research from the Damon Runyon Memorial Fund for Cancer Research (grant #DRG-918), the National Research Council of Canada (grant #731-741), and the Society of the Sigma Xi.

Phytoplankton sources of the eicosapentaenoic and docosahexaenoic fatty acids characteristic of marine metazoa. JOYCE E. DUNHAM, GLENN W. HARRINGTON AND GEORGE G. HOLZ, JR.

The most characteristic polyunsaturates of marine invertebrates and vertebrates are the $C_{20:5}$, $\Delta^5, 8, 11, 14, 17$ and the $C_{22:6}$, $\Delta^4, 7, 10, 13, 16, 19$ acids. They are members of the α -linolenic acid series, characteristic of plants, and are synthesized *via* a pathway in which methyl group-oriented desaturation of linoleic acid is a feature. Their biological origin has been a matter of concern to those interested in the sources and fates of metabolic products in marine food chains. Whales, fishes, copepods (*Calanus*) and euphausiids (*Euphasia*) contain large amounts of these acids but are unable to carry out their *de novo* synthesis. They can incorporate them intact from food, and can form them by chain elongation and desaturation of dietary 18 carbon unsaturates.

It has been assumed that components of the phytoplankton, the primary producers, synthesize $C_{20:5}$ and $C_{22:6}$ but direct proof has been offered in the literature only for 7 diatom species (rich in $C_{20:5}$) and one dinoflagellate (rich in both acids).

We have analyzed a variety of ecologically important primary producers, chrysomonads, coccolithophorids and dinoflagellates, grown axenically in a fatty acid-free medium and have found that they synthesize the polyunsaturates as follows (% total fatty acids): chrysomonads—*Isochrysis galbana* $C_{20:5}$ trace and $C_{22:6}$ 5.3%; *Monochrysis lutheri* 3.8% and 0.8%; coccolithophorids—*Cricosphaera carteri* 3.2% and 6.8%; *Coccolithus huxleyi* 2.7% and 7.3%; dinoflagellates—*Gyrodinium cohnii* trace and 35%; *Gymnodinium* sp. 20% and 20%; *Amphidinium carteri* 22% and 22%; *Exuviella* sp. 2.5% and 7.9%.

From these results it is clear that the primary producers can be important sources. The pool of $C_{20:5}$ and $C_{22:6}$ in marine fishes and in whales, then, is made up of molecules passed intact along food chains, and acids formed by conversion of simpler unsaturates by primary consumers (zooplankton) and by the vertebrates themselves.

Supported by grant AI 05802 from the National Institute of Allergy and Infectious Diseases to G. G. Holz, Jr.

Ionic fluxes in crayfish muscle fibers before and after swelling of the TTS.

PHILIP B. DUNHAM, JOHN P. REUBEN AND PHILIP W. BRANDT.

Many studies have implicated the transverse tubular system (TTS) of muscle cells in excitation-contraction coupling. Studies on crayfish and crab muscle fibers have shown that the TTS can be greatly swollen by inducing an efflux of Cl^- . The terminal portion of the membrane of the TTS is thought to have a relatively high permeability to Cl^- . By comparing the permeability properties of normal and TTS-swollen fibers of crayfish muscle, further information on the properties and function of the TTS could be obtained. Extracellular space was measured using H^3 -inulin simultaneously with the other isotopes with every preparation. In the control fibers, inulin equilibrated with 11.4% of the volume in two minutes; it equilibrated with a slower compartment over the next 8 minutes, giving a total inulin space of 16.8% at steady-state. The TTS-swollen preparations were equilibrated with inulin within one minute with a space of 19.9%. The more rapid entry of inulin and the slightly larger inulin space were expected for the TTS-swollen fibers on the basis of their morphology. The explanation for the large (6.4%) slow inulin compartment of control fibers is not clear. Rates of entry of K^{42} into fibers in steady-state were measured for control preparations, TTS-swollen preparations, and preparations equilibrated in Cl^- -free (propionate) medium. The rate of K^+ flux was the same for all three conditions. However, when rates of entry of Cl^{36} were compared for control and TTS-swollen fibers, the rate of entry of Cl^- was greater for the TTS-swollen fibers. The structural change, data on tension, and now the data on Cl^- permeability all suggest a role of Cl^- movement in the function of the TTS.

Supported by NSF grant GB-1615 and NIH grant GM 11441-03 to Dr. Dunham; NIH grant NB 05910-01A1 to Dr. Brandt; NIH grants NB 03728-04 and NB 03270 and NSF grant GB-2940 to Dr. H. Grundfest; Dr. Reuben holds an NIH Career Development Award.

Analysis of the melanin-dispersing and red pigment-dispersing hormones of the prawn, Palaemonetes vulgaris, and the fiddler crab, Uca pugilator, by means of gel filtration. MILTON FINGERMAN, ERNEST F. COUCH AND EDWARD W. STOOL.

Extracts of eyestalks from *Uca pugilator* and *Palaemonetes vulgaris*, prepared in distilled water, were passed through a column containing G-25 Sephadex. One-half- and one-milliliter fractions were collected, mixed with an equal volume of 200% sea water, and then assayed on eyestalkless *Uca* for red pigment-dispersing and melanin-dispersing activity. Some of the fractions were also assayed on intact *Uca* for red pigment-concentrating hormone. With respect to the fractions obtained with *Palaemonetes* eyestalks, although the eyestalks contained virtually no red pigment-dispersing hormone for *Palaemonetes*, they had a large dispersing effect on the red and black pigments of *Uca*. Furthermore, although both the red pigment-dispersing hormone and the melanin-dispersing hormone were retained by the G-25 Sephadex, the peak of melanin-dispersing activity never occurred in the same fraction as that which contained the greatest quantity of red pigment-dispersing hormone. Assays for red pigment-concentrating hormone revealed that this difference in peaks could not have been due to suppression of the red pigment-dispersing hormone by the red pigment-concentrating hormone in the fractions examined. Therefore, the hormones from *Palaemonetes* that dispersed the red and black pigments in *Uca* must have been different substances. In contrast to the eyestalks of *Palaemonetes*, with eyestalks of *Uca* the same fraction produced the largest response with both pigments. However, some of the other fractions had a large quantity of melanin-dispersing hormone but had virtually no effect on the red pigment. Assays for the red pigment-concentrating hormone confirmed that in *Uca* also, as in *Palaemonetes*, the red pigment-dispersing hormone and melanin-dispersing hormone were not the same substance.

Supported by Grant GB-5236 from the NSF.

Collagen from the cuticles of marine worms. LOUIS FISHMAN AND MILTON LEVY.

The compositions of the structural proteins of the cuticles of several species of marine worms were investigated. The nemertean *Cerebratulus lacteus* showed no evidence of a cuticle. Among the annelid polychaetes, *Arenicola cristata* has a secreted coat of non-collagenous material with no discernible cuticle beneath. *Nereis pelagica* has a cuticle which is difficult to remove due to the setae. This collagen could be extracted with 0.5 M NaCl and precipitated with ammonium sulfate. A partially purified collagen from *Nereis* was hydrolyzed and the amino acid composition determined. Calculated as residues per 1000 residues it contained: gly 234, pro 43, hypro 44, p-ala 30, and cyst/2 16. The rest of the amino acids were compatible with most collagens.

The sipunculate *Phascolosoma gouldi* has a firm cuticle which is almost all collagen. The collagen was not extracted by 0.5 M acetic acid, pH 4.3 citrate, or 0.5 M NaCl. It was partially dissolved by 0.5 M CaCl₂. These extracts did not show a denaturation temperature (T_D) nor did the whole cuticle show a shrink temperature but stretched at 53° C. An HCl hydrolysate gave the following amino acid composition given as residues per 1000 residues: gly 315, pro 42, hypro 64, asp 46, thre 48, ser 56, glu 128, ala 95, val 13, meth 6, isol 10, leu 13, tyr 5, p-ala 7, lys 10, hist 6, arg 90, cyst/2 8.

The structural proteins of worms should be classified as collagens because they contain hydroxyproline and are about $\frac{1}{3}$ glycine although some of the physical properties are not characteristic.

We feel that the cysteine disulfide bonds are structurally important since 0.2 M mercaptoethanol in 0.5 M CaCl₂ completely gelatinizes *Phascolosoma* cuticles.

This work was supported by NSF funds through New York University.

Reversible changes in the birefringence of the squid giant axon with temperature.

DAVID S. FORMAN.

Studies of changes in the birefringence of nerve fibers may provide information about the properties of the oriented molecules of the axoplasm. The squid giant axon is a favorable

preparation for studying this birefringence because its large retardation can be measured easily. The effect of temperature on this birefringence was studied in nine axons using a rectified polarizing microscope. Several millimeters were dissected free of surrounding small fibers, and the axon was placed in sea water on a temperature slide. Retardations were measured with a calibrated quartz wedge, and diameters were measured from photographs and with a calibrated fine focus. All measurements were made at a single region of the axon.

In the range of 5° to 40° C., lowering the temperature decreased the birefringence, and this change was completely reversible. The coefficient of birefringence, uncorrected for the contribution of the sheath, was an approximately linear function of temperature. The change in coefficient of birefringence per degree varied between axons, and averaged $+1.5 \times 10^{-6}$ per degree, with a range of 0.9×10^{-6} to 2.6×10^{-6} per degree. The coefficient of birefringence averaged $+9.5 \times 10^{-6}$ at 25° and $+6.9 \times 10^{-6}$ at 10° . After a change in temperature, birefringence reached a new equilibrium value in from 5 to 15 minutes. Good preparations gave stable values for several hours. Volume changes were negligible and were not correlated with changes in birefringence. Resting potentials were measured in four axons at the end of the experiments. These averaged 53 mv, although action potentials were found in only one of two externally stimulated axons.

The dependence of the birefringence of the axon on temperature is reminiscent of the effect of temperature on microtubular systems, such as the mitotic apparatus.

Aided by grants from the National Cancer Institute, CA 10171, the National Science Foundation, GB-5120, and by a National Science Foundation Graduate Fellowship.

Destruction of the male gonophores of Tubularia larynx by a hymenostome ciliate of the genus Paranophrys. MAURA GEENS, MARGARET JAMES AND GEORGE G. HOLZ, JR.

A *Paranophrys* has been found parasitizing the gonophores of the hydroid *Tubularia larynx*. It was located most often in male gonophores between the epidermis and the gastrodermal wall of the spadix where it fed on germ cells. It was found much less frequently in female gonophores and actinula larvae. Apparently it can attack the gastrodermis and destroy the spadix while the epidermis remains intact, since gonophores without internal structure were found packed with ciliates.

Ciliates actively divided in infected gonophores and all the individuals in a single gonophore were *Paranophrys*.

The route of infection and the physiological condition of the host at the time of infection are unknown, but heavily infected gonophores were found adjacent to uninfected ones which displayed normal, periodic muscular contractions.

Paranophrys in this circumstance is probably a facultative parasite of the hydroid. We have been able to culture the ciliate in a casein-yeast autolysate-sea water medium with a marine bacterium.

Freshly isolated from infected gonophores the ciliate was pear-shaped, had a sharply-pointed anterior end and was $28-65 \mu$ long and $17-28 \mu$ wide. Cultured ciliates were smaller; $17-38 \mu$ long and $5-18 \mu$ wide, and had a rounded anterior end. Both isolated and cultured types had 12 ciliary meridians, in contrast to the 9 of the type species of the genus, *Paranophrys marina* Thompson & Berger 1965.

We are attempting axenic culture so that the biochemistry of the ciliate can be studied. Such a DNA-rich diet as the gonophore form favors may reflect some interesting enzymatic qualities and/or capacities with respect to nucleotide catabolism.

Supported by a grant, GB-3447, from the National Science Foundation to the Department of Invertebrate Zoology, Marine Biological Laboratory, and by grant AI 05802 from the National Institute of Allergy & Infectious Diseases to G. G. Holz, Jr.

The incorporation of C-14 lysine and C-14 phenylalanine into embryonic (1-hr.) cell-free Arbacia punctulata preparations. ALBERT GROSSMAN AND WALTER TROLL.

The incorporation of C-14 lysine into a TCA-tungstate-insoluble fraction was several-fold greater than that of C-14 phenylalanine. However, C-14 lysine incorporation did not decrease

in the absence of Poly A as did C-14 phenylalanine incorporation in the absence of Poly U. *In vivo* studies showed that both amino acids were taken up into acid-insoluble material to the same extent during a one-hour incubation period immediately following fertilization. To test whether the cell-free system was capable of synthesizing a substance similar to Poly A, various concentrations of Poly U were added to the reaction mixtures to complex with any high adenylic acid-containing compounds which might have been formed. The addition of Poly U prior to the one-half-hour incubation period at 37° C. inhibited markedly C-14 lysine incorporation. It has also been shown that ATP is incorporated into an acid-insoluble fraction which is independent of other nucleotide triphosphates. It is likely, therefore, that cell-free extracts of one-hour-old sea urchin embryos are capable of synthesizing Poly A or polynucleotides similar to Poly A. Although the function of this polymer remains obscure, it could code for lysine oligopeptides which would aid in the regulation of RNA and DNA synthesis, as has been suggested for other polyamines in bacterial systems.

The cell-free sea urchin system was also found to be sensitive to exogenous sources of S-RNA. The addition of yeast S-RNA (3.7 mg./ml.) stimulated C-14 lysine incorporation but inhibited C-14 phenylalanine uptake. When *E. coli* S-RNA was used the reverse occurred; C-14 lysine incorporation was inhibited while C-14 phenylalanine uptake was stimulated. The sensitivity of this system to exogenous sources of S-RNA suggests that a change of a single S-RNA species might be a rate-limiting step in overall protein synthesis and, therefore, a possible regulator of such synthesis.

A. G. is Senior Postdoctoral Fellow of the New York Heart Association. This work was supported by Grants 71145115 and 03-013276 from the National Institutes of Health.

Isolation of surface membranes of Strongylocentrotus dröbachiensis eggs. RAYMOND L. HAYS AND ALBERT I. LANSING.

Repeated attempts at application of variations of Neville's technique for isolation of cell membranes have proven unsatisfactory when applied to some embryonic tissues and marine ova. We report herein a procedure for the isolation of surface membranes which are structurally intact, of high purity and of good yield. Eggs of *Strongylocentrotus dröbachiensis* are harvested by stimulation with 1.0% KNO₃ and suspended in an equal volume of 4.0% methylcellulose in 0.001 M NaHCO₃, pH 7.6. After 1–2 hours exposure to this solution at 4° C., the cells are washed three times in 2.0% methylcellulose and homogenized by 10–20 strokes of a loose-fitting Dounce homogenizer. The ruptured cells are then washed three times in 2.0% methylcellulose and the resulting pellet is layered over a discontinuous sucrose gradient (d 1.16, d 1.18, d 1.20). Membranes are recovered at the 1.18–1.20 sucrose interface and washed twice with 0.001 M NaHCO₃.

The membranes obtained from this procedure are smooth, homogeneous in normal section and of 200–300 Å thickness. These membranes are intact and appear as rolled sheets or sacs. Ultrastructural characteristics of membranes recovered from fertilized or unfertilized eggs are similar. No unit membrane structure is observed in either these isolated preparations or in intact eggs. The isolated membranes are of the same thickness as those of intact eggs. Similar isolations with 1.0% sialic acid yield comparable results. In both instances, the membranes recovered are intact and uncontaminated by other cytoplasmic organelles. We believe that methylcellulose may preserve the integrity of the surface membrane during the isolation procedure as does sialic acid *in vivo*.

Quantitative aspects of early life-history in the salt-marsh pulmonate snail, Melampus bidentatus, and their evolutionary significance. W. RUSSELL HUNTER AND MARTYN L. APLEY.

Few studies on growth in natural populations of invertebrates involve measures of actual organic biomass. However, a colorimetric method of "wet-oxidation" now allows assessments of total organic carbon in molluscs. Determinations of shell-lengths (L), live wet weights (WW), dry weights (DW), tissue dry weights ("ash-free") (TDW), shell calcium carbonate (Ca), and organic C content (C), were made for growth stages of the salt-marsh pulmonate, *Melampus bidentatus*. This snail, an ellobiid, retains a free-swimming

planktonic veliger, and is of considerable evolutionary interest, being almost certainly related to the stem-group of both land snails and fresh-water pulmonates.

In *Melampus*, egg-masses averaging 850 small eggs are laid after spring tides between early June and mid-July. Mean values for individual eggs are: WW = 4.72 μ g., DW = 354 nano-g., organic C = 109 nano-g. Free-swimming veligers emerge after about 13 days, and mean values per individual are: L = 127 μ , WW = 494 nano-g., DW = 129 nano-g., TDW $\approx$ 116 nano-g., Ca < 13 nano-g., organic C = 33.4 nano-g. Spat snails resettle into salt-marshes after about 6 weeks, and mean values are then: L = 675 μ , WW = 65 μ g., DW = 23.2 μ g., TDW $\approx$ 11.3 μ g., Ca = 11.9 μ g., organic C = 5.03 μ g. Comparative mean values for two size groups of adult *Melampus* are: L = 5.8 mm., WW = 36.1 mg., DW = 17.4 mg., TDW = 4.3 mg., Ca = 13.1 mg., organic C = 1.86 mg.; and L = 10.1 mm., WW = 176 mg., DW = 81 mg., TDW = 18.2 mg., Ca = 62.8 mg., organic C = 7.4 mg. Individuals can grow to L = 12.3 mm., WW = 312 mg., and organic C = 14.6 mg. The ratio of organic C in μ g. per mg. wet wt. changes from 23.1 (egg), 67.6 (veliger), 77.4 (spat) to 51.5 through 42.1 (in adult growth).

In *Melampus*, using any *real* measure, such as total organic C or TDW, growth extends through three orders of magnitude in the first three months of life, and through nearly 6 in the 3-4-year life-span. In contrast, most fresh-water or land pulmonates hatch from relatively large eggs and increase only 2 to 3 orders of magnitude in their life-span (*i.e.*, *Physa heterostropha* from 36 μ g. to 5.3 mg. organic C).

Discussions of significant reproductive adaptations in non-marine pulmonates usually emphasize "need to suppress the free larval stage," but these data suggest that selection pressures to reduce the temporal extent of immature growth have influenced evolution of larger eggs in non-marine environments.

Supported by Grant GM 11693 from the National Institutes of Health.

Histo-incompatibility and stolon overgrowth between interbreeding strains of Hydractinia echinata. FRANCES SHAPIRO IVKER.

Hydractinia echinata is normally found as a mat of tissue with protruding hydranths, growing flat on snail shells inhabited by hermit crabs. Ten strains were isolated from different shells and grown on glass slides, two strains per slide, in all possible combinations. Colonies grew in all directions and random contact was eventually made, at which time one, or, in two cases, both strains produced a tangled mass of stolons, rising as high as 5 mm. from the substrate. This stolon overgrowth eventually overran the feeding hydranths of the opposing colony. Only after the death, by starvation, of the underlying colony did feeding hydranths appear on the stolon mass, indicating one or several interacting inductive systems. During the period when a colony was actively engaged in producing overgrowing stolons, further growth and development (sexuality) in other areas was sharply reduced or halted. In two cases, a tangled stolon mass was produced between two colonies, but neither overran or destroyed the other. In all the other cases, there was a hierarchy of stolon production potency.

Several strains were sexually crossed and 40%-60% of the larvae metamorphosed on the bottom of the container. The offspring displayed either fusion with sibling colonies or histo-incompatibility and stolon overgrowth with other sibling colonies. Further tests are under way to determine the extent of incompatibility between siblings, and between parents and offspring. Crude extract of colony, and media in which incompatible strains were grown failed to produce overgrowth in a known inducible strain, suggesting that a surface-bound substance, or a heavy, low concentration compound is responsible for this histo-incompatibility.

Chromatographic studies on cardioactive compounds extracted from Mercenaria mercenaria hearts. DAVID JACOBOWITZ AND MORRIS A. SPIRITES.

Hearts of clams (*M. mercenaria*) were homogenized in 0.1 N HCl, ultracentrifuged (39,500 rpm) for 1 hour and lyophilized. Such heart extracts tested on the isolated clam hearts caused inhibition. After benzoquinonium (Mytolon) 10 μ g./cc. the heart extract caused excitation. The heart extracts were run on thin layer chromatograms, butanol/acetic acid/water (4:1:2), and dried overnight. The chromatograms were examined under UV light

and a portion was sprayed with ninhydrin. The remaining regions were eluted and tested on the clam heart. Two regions with average R_f 0.35 and 0.55 (ninhydrin-positive) showed cardio-excitatory effects (increase in amplitude and frequency). The R_f 0.35 region contained a whitish fluorescent band. The R_f 0.55 component was inhibited by methysergide (10 $\mu\text{g./cc.}$) while the R_f 0.35 eluate was not. The lower region was inhibited by trypsin (1 $\mu\text{g./cc.}$) incubation (1½ hrs. at 37° C.). The R_f for standard serotonin was 0.72. Preliminary spectrophotofluorimeter studies indicate that serotonin was not contained within the R_f 0.55 component. A third region with an average R_f 0.22 contained an inhibitory substance. This region was identical to the acetylcholine R_f . Benzoquinonium caused a reversal of effect to that of excitation. Incubation (10 minutes, 37° C.) of this fraction with washed human red blood cells did not alter the inhibitory action while the acetylcholine effect was completely inhibited by this treatment. Because of this it cannot be concluded that this heat-stable inhibitory substance is acetylcholine. There therefore appear to be at least two cardioexcitatory substances extracted from the clam heart, one of which may be a peptide, and an inhibitory substance, the chemical nature of which remains unknown.

The stalk conducting-system mediating behavioral inhibition in the hydroid Tubularia. R. K. JOSEPHSON AND J. F. UHRICH.

A *Tubularia* polyp contains two principal pacemaker systems, one localized in the distal stalk (the neck system) and the other in the hydranth (the hydranth system). Repetitive stimulation of the stalk reduces the frequency of spontaneous electrical pulses produced by these pacemaker systems. Three conducting systems are known for the stalk of *Tubularia*, the slow system (SS), the distal opener system (DOS) and the triggering system (TS). The SS, which has the highest threshold of the three, is apparently not involved in polyp inhibition, for clear inhibition is obtained with stalk stimuli well below the SS threshold. Similar elimination of the TS and DOS has not been possible, for their thresholds are very close and somewhat variable. Two other approaches, however, indicate that the TS is also not involved in polyp inhibition during stalk stimulation. (1) *Tubularia* often forms small colonies of 2-3 polyps with tissue connections between them. The TS, which is common throughout the colony, is activated each time the neck pacemaker system of any one of the connected polyps fires. Thus a polyp of a colony receives a continuous, low frequency input from the TS. But isolating a polyp from a colony does not result in an increase in spontaneous activity as would be expected if the tonic TS input were inhibitory. (2) By stimulating the distal tentacles it is possible to activate the DOS without exciting the TS. Activating the DOS alone does inhibit spontaneous activity, and distal tentacle stimulation (DOS without the TS) more fully inhibits the neck pacemaker than does stalk stimulation (exciting the DOS and TS).

Supported by USPHS grant NB 06054 and grant GB-3447 from the National Science Foundation to the Department of Invertebrate Zoology, Marine Biological Laboratory.

Intracellular ionic concentrations determined by ultra-micro flame photometry. GEORGE M. KATZ.

In standard flame photometry, an atomizer is used to introduce the sample solution into the flame. If, instead, the sample is placed on a platinum holder and inserted directly into the flame, the integral of the brief light emission will be a measure of the total number of moles of the salt on the platinum. This technique results in a thousand- to a million-fold improvement in sensitivity over standard flame photometry. An integrative ultra-micro flame photometer has been developed which can detect between 10^{-23} and 10^{-24} moles of Na and K simultaneously from the same sample. The accuracy and reliability of the method depend upon the size and shape of the platinum holder, the nature of the associated anions, interfering cations, and chemical reactions of the salts in the sample which appear to involve platinum. An analysis of these various perturbing factors has been made and techniques have been developed to minimize their effects. Thus, an accuracy of 2.5% can be achieved even in the most sensitive ranges of the instrument. The integrative flame photometer has been used to determine intracellular ions in two single cell preparations—crayfish muscle fibers and lobster axons. Single isolated muscle fibers were digested in 0.5 ml. of 0.3 M HAc for the determination of

intracellular K. The calculated concentration was 130 ± 19 mM/Kg. fiber water. One to 5 cm. of a lobster circumesophageal giant axon was placed on a platinum pan and ashed to remove the organic matrix prior to insertion into the flame. The K concentration was 348 ± 59 mM/Kg. fiber water and the Na concentration was 61 ± 18.6 mM/Kg. fiber water.

Work supported by USPHS grants NB 03728-04 and NB 03270-05 from the National Institute of Neurological Diseases and Blindness and by grant GB-2940 from the National Science Foundation to Dr. H. Grundfest.

Morphological comments on blood pressure relationships in Squalus branchial arteries. RUDOLF T. KEMPTON.

While it is quite typical to find a large drop in blood pressure during transit of a capillary bed, Burger and Bradley found in the spiny dogfish a decrement of only 1 to 5 mm. of mercury between afferent and efferent branchial arteries. Anatomical features of these gills have been examined for an explanation of this aberrant behavior.

There appears to be a low resistance to flow due to a number of factors. (1) Blood is brought into the vicinity of the secondary lamellae by an artery which is relatively short and wide. (2) Distribution from the artery to the secondary lamella is through a peculiar flattened non-elastic chamber, which extends the entire length of the filament and is much larger in diameter than the parallel artery. Each millimeter of this chamber has approximately 20 connections with the artery and supplies 20 pairs of secondary lamellae. This chamber is traversed by columns which create turbulence and which are covered with phagocytic cells. (3) Blood from this large channel flows into a thin space within each secondary lamella, one whose dimensions are approximately $1.5 \times 0.5 \times 0.01$ mm. The limiting membranes of this space are maintained in a stable position by connecting pillar cells, as described by Keys and Killmer for the teleost. (4) The large chamber and the thin but wide spaces permit blood flow with much reduced friction and without the pressure-consuming hindrance of tubes partially blocked by piled-up red cells. (5) There is a channel along the outer edge of the lamella which tends to distribute blood evenly over the entire lamella. (6) The total length of flow along the lamella is probably not more than 1.5 mm. (7) There are approximately 300,000 lamellae per animal. (8) The lamellae drain into an artery which courses along the distal end of the filament accompanied by many nerve fibers of uncertain function.

The fine structure of the redia of the trematode, Cryptocotyle lingua. PAUL L. KRUPA, ARYA K. BAL AND GILLES H. COUSINEAU.

Rediae of *Cryptocotyle lingua*, isolated from the snail host *Littorina littorea*, were studied with the electron microscope. The larvae were fixed (5% glutaraldehyde-paraformaldehyde mixture in sea water), post-osmicated, and embedded in Epon in the usual manner.

Numerous microvilli are found to extend from the epithelial surface of the intestine into the lumen. These measure about $2.3 \times 0.02 \mu$ near the anterior end of the gut, but they become shorter (0.5μ) and fewer at the blind end. The external surface of the redia is also covered by many microvilli which are located 0.06 to 0.08μ apart from each other at their proximal ends. These integumentary projections are about 0.04μ thick and some are 2.0μ long; a unit membrane (0.01μ thick) covers them. Vesicles and granules measuring up to $1.05 \times 0.6 \mu$ and mitochondria are clearly visible between the surface membrane and basement lamina. The latter is a fine-textured layer of low density, sometimes thrown into undulations, and measuring 0.1 to 0.2μ across. Directly beneath the basement lamina are found outer circular and inner longitudinal muscles. Other constituents in the redial body wall are glycogen deposits in the form of single granules (*beta* particles) and rosettes (*alpha* particles).

At one stage in its development, the integument of the immature cercaria, within the redia, is characterized by numerous irregular undulations which increase the surface area. Some of the projections may represent spine production, but others suggest that pinocytosis or phagocytosis may take place.

The presence of subsurface mitochondria and integumentary projections in this parasite supports the observations of others that the integument of trematodes is metabolically active.

Preliminary autoradiographic observations with leucine- C^{14} show that incorporation into protein occurs in specific sites of the developing germ balls and cercariae. Further electron microscopic and biochemical studies of the media and the other life-cycle stages are in progress.

This work was supported by grants-in-aid of research from the Damon Runyon Memorial Fund for Cancer Research (grant #DRG-918), the National Research Council of Canada (grant #731-741), and the Society of the Sigma Xi.

Further characteristics of lenticular gamma crystallin. SIDNEY LERMAN, WILLIAM F. FORBES AND SEYMOUR ZIGMAN.

Gamma crystallin is the cryoprotein responsible for the cold cataract phenomenon observed in the lenses of young animals. In the dogfish lens the cold cataract phenomenon is normally prevented because of a relatively high concentration of urea (0.3 *M*) but when the urea is removed from the lens by means of dialysis, an *in vitro* cold precipitation phenomenon can be demonstrated. Amino acid analyses of dogfish gamma crystallin reveal that it is similar in composition to rat gamma crystallin but that it has a higher concentration of tyrosine. Gamma crystallin from these two species does not contain tryptophan (as determined by amino acid analyses following basic hydrolysis, and by the N-bromosuccinamide reaction). Electronic absorption studies reveal an unusually high E_1^1 value (approximately 27 for dogfish gamma crystallin and 22 for gamma crystallin derived from the rat lens). This hyperchromic effect appears to be due to the tyrosine residues within the molecule. Approximately one-half of these residues can be readily ionized. It is postulated that some of the tyrosyl residues in gamma crystallin are closely held together in the native molecule, permitting a special type of electronic interaction.

Tritium-hydrogen exchange studies on gamma crystallin reveal that this protein, or some fraction thereof, contains a relatively large number of hydrogen atoms (approximately $\frac{1}{3}$ of the total) which exchange very slowly. This might indicate a relatively tight structure in at least a significant portion of the molecule. These studies also indicate that the tightest structure of gamma crystallin is at pH 4.5–4.8. Tritium-hydrogen exchange data on gamma crystallin derived from the older animal show a decrease in the number of slowly exchangeable hydrogen atoms.

Supported by USPHS grant NB 03081 and MBL ONR research grant.

Collagens of echinoderms. MILTON LEVY AND LOUIS FISHMAN.

Collagens of several echinoderms were converted to gelatins and these were analyzed for amino acid contents after hydrolysis. The purpose was to compare the compositions with those of other more familiar collagens. To prepare the materials, eviscerated sea urchins were wet-ground with mortar and pestle. Large amounts of crystalline calcium carbonate became suspended and could be poured off with the supernatant after minimal settling. After each grinding several washings were done. The grinding was repeated four times until some tendency of the residual material to float made difficulties. The residues were then suspended in water and 6 *N* HCl added, with mechanical stirring to keep the pH at 1.3–1.4. After the addition of about 20 millimoles of acid the insoluble material was collected on a cloth filter, washed with water, resuspended and the addition of acid restarted. This process was continued until no acid was required to keep the pH in the range indicated during two hours. The final solution is neutralized to pH 4, filtered on cloth, wrung "dry" and suspended in 4 times its volume of water. The suspension is heated to 120° for 3 hours, filtered and evaporated at 90° in a petri dish. The dark gelatin is scraped out for analysis. Body walls of starfish could not be ground in this way but were sufficiently softened by acid (5%) to allow blending. After further acid treatment to the endpoint used for urchins the material was suspended in water and gelatinized at 120° for 12 hours. White fenestrated ossicles were in the residue and H₂S was evident. Filtration, dialysis and evaporation gave a glassy brown residue. The analyses showed per 1000 residues 85–93 hydroxyproline, 86–94 prolines and 300–320 glycines. These are expected values. Glutamic acid, 92–108, is above vertebrate levels while lysine at 10–11 is about 40% of vertebrate gelatins.

This work was supported by NSF funds through New York University.

Transglutaminase and blood clotting. L. LORAND, J. BRUNER-LORAND, H. H. ONG, N. G. RULE AND T. URAYAMA.

Clotting of fibrinogen in vertebrates is controlled by two enzymes. Hydrolysis by thrombin uncovered glutamine sidechains to which ϵ -amino groups of lysine (and probably not α -amines of glycine as previously believed) from a neighboring molecule can subsequently crosslink by transpeptidation. By contrast, in the invertebrate *Homarus*, transpeptidation alone is sufficient for clotting. Thus crosslinking by transpeptidation seems to be the original reaction. Hydrolytic activation of fibrinogen in vertebrates merely provides an extra control.

Transglutaminase preparations (guinea pig liver) can substitute both for the transpeptidase (but not for thrombin) of vertebrate plasma and for the clotting enzyme of *Homarus*. Since transglutaminase substrates (benzyloxycarbonylglutaminyglycine (*I*) or histamine) inhibit clotting activities of the preparations, true transglutaminase specificity is involved.

Fluorescence micromethods were developed using mono(1-dimethyl-aminonaphthalene-5-sulfonyl)pentamethylenediamine (*II*) for testing transpeptidases. For example, transglutaminase, incubated with 30 mM of *I* and 5 mM of *II* (pH 8.5; 20 mM calcium chloride and glutathione) produced two additional fluorescent derivatives (*A* and *B*) which could be identified by several techniques. In paper electrophoresis (1% v/v pyridine-acetate, pH 5.4) *A* stayed at the origin, *B* migrated to the anode and *II* to the cathode. Using thin-layer carboxymethylcellulose chromatography (above pyridine solvent), *A* was stationary, *B* and *II* moved with R_f 's of about 0.4 and 0.1. On Sephadex G-10 column (water solvent), *A* was eluted first, then *B*; *II* was greatly retarded. Excitation and emission spectra of *A* were different from those of *B* and *II*; the latter two were identical. Acid hydrolysis of *B* yielded glutamic acid and glycine, equimolar to the naphthalene chromophore present (computed from absorptivity at 326 m μ). Properties of *B* were those expected from the γ -amide of *I* with *II*. Product *A* was the result of incorporating *II* into the proteins of the enzyme preparation.

Aided by USPHS Research Career Award.

Vinblastine and griseofulvin reversibly disrupt the living mitotic spindle. STEPHEN E. MALAWISTA AND HIDEMI SATO.

Studies of particular stages of mitosis are hampered by the rapid passage of most dividing cells through the mitotic cycle. However, freshly spawned, unfertilized oocytes of the marine annelid, *Pectinaria gouldi*, persist for several hours at the first meiotic metaphase. The content of oriented spindle material in these oocytes can be measured in polarized light as retardation induced by spindle birefringence. We have studied spindle effects of some metaphase-arresting agents that are of special medical interest.

Perfusion with the chemotherapeutic *Vinca* alkaloid, vinblastine (Velban, 1×10^{-5} M), resulted in a decrease in birefringence and size of the spindle and, within 6 to 12 minutes at 24° to 25° C., complete dissolution. On subsequent perfusion with artificial sea water, recovery of spindles began in about 20 minutes and was complete by 40 to 50 minutes. This reversible effect was repeatable in the same preparation, and was at least as efficient as that of N-desacetyl-N-methylcolchicine (Colcemid, 1×10^{-5} M). Neither dissolution nor recovery was affected by glutamate, 1×10^{-3} M. Dissolution by vinblastine was retarded in 40% D₂O-sea water, but recovery was not hastened by D₂O-sea water. At 1×10^{-5} M, vincristine (Oncovin), a *Vinca* alkaloid of similar structure, did not abolish spindle birefringence. At 1×10^{-4} M, dissolution required 24½ minutes, and recovery was incomplete, with many small tri- and tetrapolar spindles. Podophyllotoxin (1×10^{-7} M) produced spindle dissolution in about 20 minutes; recovery was incomplete.

Perfusion with the fungistatic antibiotic, griseofulvin (Grisactin, 1×10^{-5} M), resulted in a decrease in spindle birefringence and size, disappearance in 3½ to 6½ minutes, and complete recovery in 5½ to 11 minutes. This reversible dissolution could be carried out repeatedly in a single oocyte; recovery was complete even with a 10-fold increase in concentration of griseofulvin.

These agents, then, are useful additions to the colchicine analogues for studying the molecular architecture of the mitotic spindle. Furthermore, the shift of emphasis from

"mitotic arrester" to "spindle poison" will hopefully stimulate the investigation of possible effects of such substances on gelated structures in resting cells.

Aided by grants from the National Cancer Institute, CA 10171, the National Science Foundation, GB-5120, and the National Institute of Arthritis and Metabolic Diseases, AM 10493. Dr. Malawista is a Senior Investigator of the Arthritis Foundation.

Inland culture of Botryllus schlosseri; genetic crosses. ROGER MILKMAN AND JUDITH PEDERSON.

Botryllus schlosseri has been cultured in beakers of still Instant Ocean containing initial daily concentrations of $0.5\text{--}2.2 \times 10^5$ cells/ml. of *Cyclotella nana*, a centric diatom about 5 micra in diameter. Asexual reproduction is rapid, with zooids often producing 4 buds apiece, all of which become functional zooids. Tadpoles give rise to egg-producing colonies in less than $1\frac{1}{2}$ months; once begun, egg production has improved with each asexual generation. Colonies must be kept vertical, upside down, or in between.

Techniques for *in vitro* fertilization and embryo culture, necessary for the control required in the genetic crossing of self-compatible hermaphroditic organisms, have been refined. Up to 100% of eggs nested in a dense layer of sperm are fertilized; good yields of metamorphosing larvae are obtained when embryos are placed on filter paper in fingerbowls and transferred daily. Crosses to date support Sabbadin's conclusion that double pigment bands are associated with a simple dominant gene; in one locale (Eel Pond) such a gene's frequency is calculated to be 0.4. The work preliminary to the extensive investigation of the developmental genetics of *Botryllus* is now complete.

Supported by Research Grant GM 07810 from the National Institute of General Medical Sciences.

Hyperbaric oxygen and succinic dehydrogenase in the embryology of Tubularia. JAMES A. MILLER, JR., DAVID L. DESHA, PAUL M. HEIDGER AND FAITH S. MILLER.

Increases in the activity of succinic dehydrogenase accompany each visible step in morphogenesis in *Tubularia* (Miller, Hegab and Miller, 1964). Since oxygen stimulates regeneration (Barth, 1938) but hyperbaric oxygen inactivates succinic dehydrogenase (Stadie and Haugaard, 1954), a study was made to determine whether hyperbaric oxygen was inhibitory or stimulatory in the embryonic development of *Tubularia* and what its effects were on succinic dehydrogenase activity. Hyperbaric oxygen at all levels tended to protect embryos and larvae from death in standing sea water. Oxygen at $1\frac{1}{2}$ atmospheres absolute did not affect embryonic development but at 2, $2\frac{1}{2}$, 3 and 4 atmospheres blocked development at early actinula stages. Blocked embryos were unable to secrete adherent material from their adhesive organs or produce perisarc and thus were unable to transform into polyps. The block in differentiation developed between 24 and 48 hours and was completely reversible even after 72 hours at 4 atmospheres.

Succinic dehydrogenase was estimated histochemically by a modification of the Nachlas method. It showed a reduction in overall activity in embryos subjected to hyperbaric oxygen and the failure to develop the localized zones of high activity which were associated in controls with differentiation of tentacles, gonophore buds, perisarc-secreting region and adhesive organ. Since hyperbaric oxygen does not inactivate a variety of enzymes including cytochrome oxidase but does reversibly inactivate those containing SH groups including succinic dehydrogenase, our experiments suggest that enzymes with SH groups are concerned with differentiation in *Tubularia* and that additional studies along these lines will further elucidate the energetics of development in this species.

The effect of phenylthiourea on melanogenesis in the embryo of Fundulus heteroclitus. BEVERLY S. MITCHELL AND GEORGE SZABÓ.

Phenylthiourea in a concentration of 8×10^{-3} M was observed to inhibit melanin formation in the yolk sac epithelium, on the body surface and in the retinal pigment epithelium of

Fundulus embryos. Organ differentiation continued in those embryos developing in PTU, although the growth rate was impaired and maximal survival time was nine days.

Electron microscopic studies of the pigment layer of the normally developing retina showed a number of fully melanized granules (melanosomes) surrounded by a single, closely applied membrane. In contrast, the retinal pigment layer of embryos which developed in PTU for three days prior to fixation and in which macroscopic deposition of melanin had been inhibited, showed very few melanosomes, mostly in pre-melanosome stage. There were also large vacuolated areas containing some pigment, presumably early pre-melanosomes. In addition, there were large numbers of glycogen granules present. Cell divisions were observed to be taking place after PTU treatment as well as in the controls.

Preliminary studies on the incorporation of tritiated thymidine, valine and DOPA into embryos developing normally and in PTU were carried out. The effect of PTU on thymidine and valine incorporation was variable, giving little indication of the overall effect on growth and development. PTU was consistent, however, in decreasing the incorporation of tritiated DOPA into the entire embryo and into the developing eye.

The morphogenetic evidence, in conjunction with these biochemical studies, indicates that PTU interferes with the melanization of the preformed melanosomes. This is consistent with the view that PTU is acting directly on the tyrosine-tyrosinase system.

Supported by a Student Fellowship of Harvard Medical School and by a Research Grant #CA 050401-07, National Cancer Institute, USPHS and a Career Development Award #Kc-GM-14.987 USPHS.

Distribution and responses to salinity of larval chironomids from the upper Pocasset River. SAMUEL C. MOZLEY.

The sandy-bottomed upper 120 meters of the Pocasset River harbor the larvae of eight species in three sub-families of the dipteran family Tendipedidae (=Chironomidae). Salinities in this region vary from 0.5 to 26 ppt. Salinities of interstitial water also vary tidally to a depth of 9-10 cm., where they remain at 4-8 ppt. The tidal salinity range decreases steadily to this depth. Springs affect this pattern at some sites.

The two most abundant species are *Tendipes modestus* (Say) and *Polypedium scalaenum* (Schrank). The former is denser subtidally, and the latter, intertidally. These and four other species extend to the edge of silty bottoms downstream, but two species' larvae are restricted to the upper 30 meters. *T. modestus* occurs as deep as 4 cm. into the sediment, but is most abundant in the top 2 cm. Larvae of this species build sand-and-mucus tubes, which they irrigate periodically by undulating. In the laboratory, intervals between undulation are longer at high salinities. A greater percentage of undulation time is spent pumping interstitial water out through the tube opening with increasing salinity to 19 ppt., by larvae in tubes with only one opening.

The LD-50 time for *T. modestus* at 42° C. is not affected by salinity. At 41°, salinities greater than 6 ppt. decrease the survival time, up to 32 ppt. Here the LD-50 time is the same as at 42°. At 40°, a salinity of 48 ppt. is required to reduce the LD-50 time to this point. Reduction in available oxygen decreases tolerance to high salinities.

This work was supported by NASA training grant NsG(T) 123-64 and NIH grant 2TIGM 535-06 to the Marine Ecology Course of the Marine Biological Laboratory. Special appreciation is due Dr. Howard L. Sanders for regular advice during this study.

The development of an ordered array of collagen in Fundulus. JOSEPH B. NADOL, JR.

The skin of *Fundulus heteroclitus* possesses a highly ordered array of collagen fibrils, called the basement lamella. Fibrils are arranged in alternate layers nearly parallel to the surface and crossing each other at 105-110°. Previous investigators have drawn an analogy between the basement lamella of amphibian skin and the structure of plywood, and have suggested that each fibril layer is exactly parallel to the basement membrane. They have further proposed that during growth the basement lamella is established by polymerization of collagen fibrils at the basement membrane in one orientation and that, by some repeated

switching mechanism, each subsequent layer is laid down at approximately right angles to the previous (and now underlying) layer.

Our observations in *Fundulus* do not support this "plywood theory." Instead collagen layers descend at a slight angle from the basement membrane. The name "scindulene" has been adopted to describe this shingle-like arrangement of layers. As a mechanism of establishing the orthogonal array of the basement lamella, it is proposed that collagen fibrils are inserted at end points into the basement membrane and are polymerized in one orientation only in one area of the basement membrane, and at 105–110° to this first orientation in adjacent areas, thus obviating the necessity of a switching mechanism required by the plywood theory. Thickening of the basement lamella with growth is accounted for by expansion of these "areas of insertion" of fibrils into the basement membrane. As a result the number of fibril rows in each layer, but not the number of layers, increases. This corresponds exactly with the observed morphological changes with growth in *Fundulus*.

Other morphological findings in *Fundulus*, such as the appearance of "crossover fibrils" continuous from one layer to the next in the same orientation, and the gradual tapering and disappearance of the basement lamella toward the fin tip, are more easily explained by the scindulene theory than by the plywood theory.

This work was supported by Grant 5T1-GM-707 from the National Institutes of Health to Dr. Keith R. Porter and by Grant GY 108 from the National Science Foundation to Harvard University and the author.

The diurnal nature of the tidal migration rhythm of the diatom, Hantzschia. JOHN D. PALMER AND FRANK E. ROUND.

The intertidal benthic diatom, *Hantzschia*, lives buried in the sand at night and during high tide, but emerges onto the surface sediments during daytime low tides. The cells accumulate in such vast numbers on the surface, that the exposed sediments are colored a golden brown.

Intact *Hantzschia*-bearing sand samples were returned to the laboratory where the rhythmic movements could be studied under controlled conditions. In constant temperature, continuous illumination of a constant intensity, and away from the influence of the tide, the rhythm was found to persist for as long as 11 days. During this time the diatoms appeared on the surface in approximate synchrony with the feral cells on the sand-flats. A similar laboratory population behaved in a like manner in alternating light and dark conditions (14.5 hrs. light; 9.5 hrs. dark).

In nature, as the time of low tide moves to the late afternoon, the following low tide overlaps with sunrise the next day; thus twice each month the sand-flats are exposed both in the morning and late afternoon. During these days the diatoms gradually abandon the afternoon phase of their rhythm and now appear on the surface during the early morning exposure. To examine this phase change in detail, cells were collected during late afternoon tides and placed in either alternating light-dark conditions, or under constant light in the laboratory. Over the next three days, in both conditions, the rhythm rephased to the morning hours, indicating that this abrupt phase change is also under clock control.

Using these results a working hypothesis was developed, postulating that the rhythm is a manifestation of an interacting dual-clock system: one clock running at a speed of 24.8 hours per day and responsible for a bimodal migration rhythm; and a second solar-day clock responsible for the suppression of the night-time supra-surface phase of the migration rhythm.

This work was supported by National Science Foundation grant GB-5045 to JDP.

Intracellular distribution of malic dehydrogenase isozymes in developing red and white halves of sea urchin eggs. GRANT PATTON, LAURENS METS AND CLAUDE VILLEE.

Whole unfertilized eggs of the sea urchin, *Arbacia punctulata*, have five to seven electrophoretically distinct forms of L-MDH, which decrease to three in the 64-cell stage. Centrifugal separation of these embryos into large and small blastomeres revealed two forms of the enzyme in the former and three in the latter fraction. Since inhibition of protein synthesis

with actinomycin and puromycin did not alter this change it was of interest to investigate other cytoplasmic influences present at fertilization.

Red and white cell halves were obtained by centrifuging whole unfertilized eggs in a sucrose gradient. The lighter, white half, containing nucleus, microsomes, mitochondria and a little yolk, had the full complement of MDH isozymes identified by polyacrylamide gel electrophoresis of whole eggs. At the two-cell stage following normal fertilization this number was reduced to four. The heavier red halves, which contain yolk, pigment, some mitochondria and soluble enzymes, contained five of the seven isozymes found in whole eggs and white halves. Ninety minutes after fertilization red halves had lost two forms of MDH and showed increased concentration of the early cathodal band. The average ratio of the rate of reaction of MDH with acetyl pyridine adenine dinucleotide (APAD) compared to NAD was 0.55 in white halves and increased to 0.61 in two-cell embryos. In unfertilized red halves the ratio was 0.68, increasing to 0.99 in the samples 90 minutes after fertilization.

Soluble and particulate MDH was prepared by differential centrifugation at 22,000 *g* for 20 minutes. Four soluble isozymes (analogue ratio 0.55) and four particulate isozymes (analogue ratio 1.51) were found; only one of the particulate forms coincided with the soluble isozymes. The seven isozymes of the egg thus reflect a synthesis of soluble and particulate forms.

Arbacia sperm revealed three forms of L-MDH, one identical with the darkest anodal particulate isozyme and two intermediate bands characteristic of the soluble fraction; the analogue ratio of the total sperm sample was 2.30.

Autoradiographic studies of DNA synthesis in cultures of peripheral blood from the smooth dogfish, Mustelus canis. THORU PEDERSON AND SEYMOUR GELFANT.

We have designed a culture method for peripheral white blood cells from the smooth dogfish. Whole blood is drawn from the caudal vein and allowed to sediment in the syringe at 37° C. The white blood cell-rich supernatant plasma is extruded into a sterile tube and a cell count is made. From 50 ml. of whole blood this method routinely yields around 5×10^8 cells. The cultures are seeded at 2×10^6 cells/ml. The culture medium is elasmobranch Ringer's without Ca^{++} (to prevent agglutination *in vitro*) and with 0.01 gm./L. phenol red as a pH indicator (pH adjusted to 7.8 with 0.1 N HCl or NaOH). Just before use the medium is supplemented with penicillin (0.06 gm./L.), streptomycin (0.13 gm./L.) and autologous plasma (20% v/v). Cultures are put up in screw-cap vials and are kept stationary at 20° C. With this method, cells remain viable for at least 25 days.

The basic question we sought to answer was whether or not these cells move through the cell division cycle *in vitro*. No mitoses were observed in any of several experimental situations, including the use of factors which are mitogenic for human lymphocyte cultures. Nevertheless, dogfish white blood cells do synthesize DNA and RNA *in vitro*, as measured by autoradiography of cells treated with TdR- H^3 or UR- H^3 , respectively (at 2 $\mu\text{c.}/\text{ml.}$). Hemacytoblasts and small lymphocytes are most active in these respects. However, *in vivo* experiments using pulse administrations of TdR- H^3 (at 1 $\mu\text{c.}/\text{gm. body wt.}$) indicated that peripheral white blood cells are not engaged in DNA synthesis.

These results suggest that dogfish peripheral white blood cells are in the G_1 period of the cell cycle *in vivo*, are stimulated to enter the S period *in vitro* and then become arrested in the G_2 period.

This work supported by research grant GB-2803 from the National Science Foundation.

The effects of NaCl on respiration of Squalus acanthias rectal gland in vitro. JACOB RAAB.

Burger (1960) showed that *Squalus acanthias* rectal gland has an ion-regulating function. The following results indicate a NaCl-induced humoral control of rectal gland metabolism.

Blood was drawn from the caudal vein of a normal fish and immediately 10 ml. of 1, 2, or 4 M NaCl were injected into the same fish and 10 ml. of blood were again drawn from the fish after one hour. The sera obtained were used for incubation media. Rectal gland tissue was prepared by slicing the gland freehand with a razor on Ringer-moistened filter

paper at 0° C. and quickly transferring slices to Ringer's (Na, 250 mM; K, 10 mM; Ca, 5 mM; Mg, 5 mM; Cl, 270 mM; SO₄, 5 mM; glucose, 10 mM; Tris, 10 mM; malic acid, 4.4 mM; urea, 400 mM) at 0° C. Constant pressure respirometers were shaken at about 140 oscillations per minute in a stirred water bath that varied from 18° to 21° C. The tissue and incubating fluid, under air, were allowed to equilibrate for one hour; readings were taken for the next two hours.

Respiration (in microliters of oxygen consumed per hour per mg. dry weight of tissue) in Ringer's was 1.79 (18 determinations), 2.32 (21) in normal serum, and 2.72 (15) in serum from the salt-loaded fish. These incubation media had little effect on tissue levels of Na, K, or Cl. Incubation in serum from salt-loaded fish in the presence of 10⁻⁴ ouabain led to increased tissue Na and decreased tissue K, and resulted in an oxygen consumption of 2.31 (7). Raising the NaCl and osmotic pressure of the control serum to that of the serum from salt-loaded fish led to a respiration level of 2.35 (7). Enhanced respiration occurred with serum from an injected fish which had the same K level as control serum. Little difference was found in the respiration level of spleen tissue slices incubated in serum from control or salt-injected fish.

Supported by USPHS Grant GM 10542.

Fine structure of intercellular contacts in the sponge, Microciona prolifera. JEAN-PAUL REVEL.

While much information on cellular adhesion has been gained by the study of the reaggregation of sponge cells, there are as yet few data on the ultrastructural aspects of cell-to-cell relationships in these organisms. In the preliminary work outlined here, we describe the intercellular junctions in the intact sponge. *Microciona* was most successfully preserved by fixation in mixtures of glutaraldehyde and formaldehyde followed by osmium tetroxide. The cell membrane of the cells of *Microciona* presents a very clear "unit membrane" appearance. We could find no evidence for desmosome-like structures, nor for the presence of septate junctions. There are, however, areas where cell membranes of adjacent cells run an approximately parallel course, separated by a space rarely wider than 1000 Å. In sponges fixed under conditions producing extreme shrinkage, as judged by the distortion of the histological organization, one finds short pseudopodia extending from one cell toward its neighbor. There seems to be a focal area of contact between the adjacent membranes, a small macula occludens, at the apex of the pseudopod. In sponges where the histological appearance is more normal, the blunt cytoplasmic projections are rare, and one finds instead wide areas of contact with the typical appearance of a tight junction: there is a complete obliteration of the extracellular space which results in the formation of pentalaminar junctions. One also finds areas where a narrow gap, only 10 Å or so wide, is present between the facing outer leaflets of opposing membranes. Both types of junctional specializations are found between similar and dissimilar cells. A single examination of reaggregating sponge cells shows cell contact specializations similar to those observed in the intact organism. It would thus appear that structures similar or identical to the tight junctions of vertebrates are already present in organisms that are still at the "cellular level of organization."

Supported by grant GM 11380 of the USPHS.

Determination of the quantum efficiency of the human eye by a new method. GEORGE T. REYNOLDS.

The quantum efficiency of the human eye has been measured over a period of years with contradictory results. The efficiency has usually been determined by comparing the performance of a human eye with that of an ideal device in which all of the light entering the eye results in information processed for the performance of the prescribed task (Rose, 1942, 1946; Jones, 1957; Barlow, 1962). The quantum efficiency of the eye is then given by the ratio:

$$Q = \frac{\text{Quantity of light required by ideal device to perform task}}{\text{Quantity of light required by human eye to perform task}}$$

The value of Q is found to depend upon the level of light to which the eye is adapted, and may also depend upon the task to be performed.

The present determination was made by comparing the performance of the eye with that of an image intensifier cathode of known quantum efficiency (Reynolds, 1964). The task was to detect a pattern in a background of noise (Rose, 1946). In one case the eye was required to detect the pattern unaided, and in the other case was required to detect the pattern at the output of the image intensifier system. The ratio of the light levels required in the two cases provides a value for Q . Measurements were made on five observers, with eyes adapted for fluxes ranging from 0.5×10^6 to 4×10^6 quanta $\text{sec}^{-1} \text{deg}^{-2}$, under conditions in which 10^3 to 10^5 quanta entered the eye during the integrating time. Results were in excellent agreement with those obtained by Barlow (1962) under similar conditions, but by an entirely different method, and gave reproducible values for Q of between 10^{-3} and 10^{-4} .

Supported by AEC Contract AT(30-1)-3406.

The identity and photon yield of scintillons of Gonyaulax polyedra. GEORGE T. REYNOLDS, J. W. HASTINGS, HIDEMI SATO AND A. RANDOLPH SWEENEY.

Light emission in *Gonyaulax polyedra* originates from particles which are obtained in active form by gentle rupture of cells in pH 8.2 buffer. These light-emitting bodies, termed scintillons, emit a brief (0.1 sec.) flash of light *in vitro*, when the pH is simply lowered to 5.7.

In the experiments reported here we have used the image intensifier, together with the polarizing microscope, to observe and record the *in vitro* flash from individual scintillons and we have determined that each emits about 500 photons. This confirms the conclusion that the scintillon is to be identified with the birhombohedral crystalline structure which occurs in the purest preparations, for previous measurements of the average photon yield per crystal have also been about 500. These measurements thereby exclude the possibility that the scintillon could be some other structure emitting many times as much light per particle, but occurring less commonly. The results also exclude the possibility that only a small percentage of the structures in purified preparations are active scintillons.

In order to observe the light from individual scintillons, they must be kept virtually immobile while the buffer is rapidly replaced by another at a lower pH. This was achieved by taking advantage of the fact that scintillons strongly adhere to a Butvar film on a coverslip. A scintillon suspension was placed in a 1 mm. space between slide and coverslip for 4 minutes and then repeatedly flushed with pH 8.2 buffer, and the adhered scintillons were counted as strongly birefringent bodies. While appropriately positioned on the microscope stage, 0.03 N HAC was rapidly flushed under the coverslip utilizing a pneumatically driven syringe. The scintillons remained affixed during this treatment. The flashes were observed through the microscope and image intensifier and recorded photographically.

Supported in part by AEC Contract AT(30-1)-3406.

Behavioral sequences in the feeding response of Hydra littoralis. NORMAN B. RUSHFORTH AND FLORENCE HOFMAN.

The capture and engulfment of a single *Artemia* nauplius by *H. littoralis* consists of a series of complex behavioral sequences: (1) nematocyst discharge, (2) tentacular movements, (3) mouth opening, creeping over the prey and closure, and (4) inhibition of column contractions. We wish to report some factors involved in tentacular movements and inhibition of column contractions.

On attachment of the prey to a tentacle by nematocysts there is a latent period (mean 2.9, s.d., 3.0 sec). Then the portion of the tentacle proximal to the *Artemia* contracts, sometimes accompanied by oral bending or inward spiraling when the prey attaches to the basal or distal tentacle regions, respectively. The latency is independent of the position of attachment or the type of tentacular movement. This implies that neither conduction time nor the time for the diffusion of chemical factors to hypostomal regions is a predominant component of the latent period.

As the prey nears the mouth on tentacle contraction, the surrounding tentacles concertedly flex orally (a concert). Concerts are frequent during and following engulfment of the prey, while tentacle and column contractions are inhibited. They are highly coordinated movements unlike tentacle writhing which is also frequently observed when hydra feeds. Concerts are

a component of endogenous behavior, whose frequency is markedly enhanced by exposure of hydra to homogenates of *Artemia* or reduced glutathione (GSH). Concert frequency increases approximately linearly with log GSH concentration, over the range 1×10^{-10} to 5×10^{-9} M GSH, above which tentacle writhing is induced. No electrical correlates of concerts have been observed.

Endogenous, compound, electrical pulses (approx. 30 mV, and 100 msec. duration) and their associated column contractions are inhibited when hydra is exposed to *Artemia*, homogenates of *Artemia* or reduced glutathione. Contractions and associated electrical pulses recorded from excised tentacles are also inhibited by these stimuli.

Supported in part by grants from the National Science Foundation and the National Institutes of Health.

Recovery from radiation-induced mitotic delay in sea urchin eggs. RONALD C. RUSTAD.

Sea urchin eggs were cut into halves. Following fertilization with normal sperm the whole cells and the diploid half-cells divided at the same time, while the haploid half-cells were delayed from 5 to 15 minutes. The mitotic delay arising from γ -irradiation of the sperm was greatest in diploid half-eggs, less in diploid whole eggs, and least in haploid half-eggs. The mitotic delay in each type of cell was dose-dependent over the range of 10 to 50 Kr.

Radiation-induced mitotic delay may depend not only on the initial damage but also on the amount of recovery that can occur before a critical mitotic stage. Other studies in our laboratory have demonstrated that haploid eggs remain U.V.-sensitive longer than diploid ones. Therefore, the critical radiation-sensitive mitotic stage appears to be delayed in haploid cells. Such a delay would allow more time for recovery in haploid cells than in diploid ones. If the rate of recovery is dependent upon the cytoplasmic volume, diploid half-cells would be expected to divide later than diploid whole cells. The volume dependence may be associated with the capacity for protein synthesis. The rate of prefertilization recovery of γ -irradiated eggs has previously been shown to be reduced by exposure to puromycin (an inhibitor of protein synthesis). Since unfertilized *Arbacia* eggs do not normally synthesize proteins, the puromycin effects suggested that radiation activates protein synthesis. Preliminary autoradiographic evidence indicates that γ -irradiation stimulates the incorporation of H^3 -leucine into the proteins of unfertilized eggs.

These data are compatible with the hypothesis that γ -ray-induced mitotic delay is inversely proportional to the time available for a recovery process, the rate of which is dependent on cytoplasmic volume and perhaps specifically on protein synthesis.

These studies were supported by the U. S. Atomic Energy Commission and the Office of Naval Research.

An analysis of living squid sperm head fine structure through polarized UV microbeam irradiation. HIDEMI SATO AND KENNETH J. MULLER.

Living, mature sperm heads of the squid *Loligo pealeii* were irradiated after the method of Inoué and Sato with a UV microbeam plane-polarized at various angles to the main sperm axis. With reduction of birefringence upon irradiation, a regionally constant shift of the optic axis toward the polarized microbeam's E-vector was observed. Measured optic axis shifts were 8° in the anterior and posterior 2μ and 12° in the middle 3.5μ of the sperm head. The angles between the optic axis and main axis of the sperm in the three regions are -3° , $+2^\circ$, and $+8^\circ$ to $+20^\circ$ progressing posteriorly, viewed with maximum head curvature. DNA absorbs energy differentially with the UV E-vector polarized in and normal to base planes. From our data we obtain an arrangement of DNA which deviates base plane normals from the optic axis by $\leq 8^\circ$ in posterior and anterior regions and $\leq 12^\circ$ in the middle.

The observations are consistent with a model with B-form DNA molecules bundled in helices of 8° pitch from their main axes, and arranged longitudinally during spermiogenesis. In the model, each helix represents a chromonema and is paired, forming a double-helical chromosome with chromonemata separated by less than the helix diameter. With equal gyre periods, the chromosomes can mesh when aligned, becoming unresolvable. Placing

shorter chromosomes posteriorly and anteriorly, we may envision a twist of 4° in the middle region, explaining regional variation in optic axis shifts. This model agrees with measured birefringence of -2×10^{-2} , with Wilkins' x-ray diffraction studies indicating vertical B-form DNA in squid sperm, and with visual interpretations of phase, polarization, and electron micrographs during spermiogenesis. Other models, with grounds for rejection, shall be discussed.

Aided by grants from the National Cancer Institute CA 10171 and the National Science Foundation, GB-5120.

The effect of D₂O on the mitotic spindle. HIDEMI SATO, SHINYA INOUÉ, JOSEPH BRYAN, NORA E. BARCLAY AND CHRISTOPHER PLATT.

The effect of D₂O has been studied in the first meiotic metaphase spindles of *Pectinaria gouldi* oocytes and in the dividing eggs of *Arbacia punctulata* and *Lytechinus variegatus*. Polarization microscopy reveals that the volume of the spindle increases 8 times, the retardation 2 times while the coefficient of birefringence remains constant at 3×10^{-4} . The increase in volume and retardation is a function of D₂O concentration and the stage of mitosis at which it is applied. Maximum increases occur with 45% D₂O and application during metaphase. These changes are rapid, being 80% complete within 40 seconds. The effect of D₂O is completely reversible. It is not a secondary effect brought about by change of acidity, even though $pD \approx pH + 0.4$. The medium can be varied from pH 7.2 to 8.6 with no detectable change in spindle size, shape, or retardation.

The constant coefficient of birefringence suggests that there is an increase in (microtubule(?)) protein in deuterated spindles. To measure this increase, spindles from both normal and 40% deuterated *Arbacia punctulata* eggs were isolated in 1.0 M hexylene glycol. Such isolated spindles show volume and retardation increases equivalent to those observed *in vivo*. The isolated spindles were dissolved in 0.6 M KCl and analyzed in the ultracentrifuge using Schlieren optics and a UV scanner. The major 22S protein component of Kane and Stephens is observed in both preparations. The amount of this 22S protein/spindle increases 2.6–10 times in the deuterated, isolated spindles. This increase agrees with the predicted values obtained from polarization microscopy.

Purified 22S protein was obtained from normal and deuterated unfertilized *Arbacia* eggs. The amount of 22S protein in both samples is essentially equal, suggesting a constant pool of 22S protein in the whole egg. Thus the quantity of 22S protein in the mitotic apparatus is enhanced by D₂O.

Aided by grants from the National Cancer Institute, CA 10171 CB, and the National Science Foundation, GB-5120.

Mercenene: Preliminary analysis of induced focal changes in the Krebs-2 carcinoma fine structure. SISTER M. ROSARI SCHMEER, O.P., AND REV. J. D. CASSIDY, O.P.

Cytological effects of Mercenene on the Krebs-2 tumor *in vivo* were surveyed. The dearth of published research on the ultrastructure of this carcinoma presented a major difficulty. Mercenene was extracted from the clam *Mercenaria*, purified, and administered to 4–5-week-old female CF1 mice implanted with the Krebs-2 carcinoma. The 7-day treatment was followed by autopsy and tumor biopsy for one-half of the treated animals and for all the untreated mice. The remaining 45 treated animals were maintained for a 6-month longevity study. Carcinoma tissue from treated and control mice, and biopsied tumors from the longevity group, were then prepared for ultrastructural investigation. Fine structure analyses utilized four parallel experiments with complementary methods of cytological preparation. Three gave excellent and reproducible results: (1) Millionig's sodium phosphate-buffered OsO₄; (2) sodium phosphate-buffered 5% glutaraldehyde, and OsO₄; (3) s-collidine-buffered 5% glutaraldehyde and OsO₄. Thin sections of an Epon-Araldite mixture were viewed in the Siemen's Elmiskop I. Cytochemical techniques utilizing azure II-methylene blue, azure B, basic fuchsin, and Nile blue A were conducted on alternate 1-micron sections. No changes were detected in untreated cells upon examination of the nuclei, nucleolar apparatus, membranes, Golgi zone, mitochondria,

endoplasmic reticulum, and RNP particles. In Mercenene-treated tumor cells changes were observed in polysomes, free ribosomes, the nuclear envelope and nucleolar apparatus. Quantitative alteration at these sites by Mercenene suggests possible antagonism of protein metabolism in the cancer cells under these experimental conditions. Induced oncolysis, reflected in fine-structure changes, could further elucidate the antitumor activity of Mercenene. Correlated cytochemical investigations have been initiated to test this hypothesis.

Supported by American Cancer Society Grant T-361, NSF Fellowship 73182, USPHS Grant GN 296, Ashland County Health Foundation, Ashland County Cancer Society.

Bottom temperature and faunal provinces: continental shelf from Hudson Canyon to Nova Scotia. THOMAS J. M. SCHOPF AND JOHN B. COLTON, JR.

In open marine waters, temperature and substrate are the biologically important factors that change most radically and thus have marked influence on the distribution of benthic organisms. Bottom temperature regimes reported here were determined from approximately 5000 measurements collected on 29 cruises of Bureau of Commercial Fisheries ships, mostly in 1955, 1956, 1963, 1964 and 1965. From 1 to 8 cruises cover each month; all but two months (January and June) are represented by data from three or more cruises.

Quarterly (March, June, September and December) average bottom temperatures are as follows: Nantucket Shoals, (20-80 m. depth), 3.6°, 7.5°, 11.3°, 9.5° C.; Georges Bank (4-100 m. depth), 4.2°, 8.6°, 13.0°, 8.3° C.; Gulf of Maine (100-377 m. depth), 4.8°, 5.9°, 6.7°, 6.1° C.; Browns Bank (40-100 m. depth), 2.5°, 4.5°, 7.3°, 5.0° C.; Nova Scotia Shelf (40-140 m. depth), 2.2°, 4.6°, 7.0°, 4.6° C. The subsidiary influence of continental slope water, which sometimes rises onto the continental shelf, is not considered here.

Most striking is the similarity of bottom temperatures on Georges Bank and Nantucket Shoals, and the distinctly lower temperatures on nearby Browns Bank. The Gulf of Maine is intermediate in its temperature characteristics. This temperature distribution is apparently related to bottom currents that circulate water over the Nova Scotia Shelf and Browns Bank, through the Gulf of Maine, and then onto Georges Bank. The Nantucket Shoals appear to obtain bottom water from both Georges Bank and the Gulf of Maine. These different temperature regimes suggest inclusion of Georges Bank and Nantucket Shoals in the same faunal province distinct from the Gulf of Maine, Browns Bank and the Nova Scotia Shelf.

Supported in part by a grant from the Ford Foundation to the MBL Systematics-Ecology Program.

*Evidence against the presence of functional pigment-dispersing nerve fibers in the sand flounder *Scophthalmus aquosus*.* GEORGE T. SCOTT AND K. K. WONG.

We conclude that only pigment-aggregating nerve fibers are active in the sand flounder on the basis of the following observations: (1) Nerve sectioning or blocking by pressure or cold produces only pigment dispersion. Electrical stimulation of sectioned nerves results in blanching. (2) A negative "Parker Effect" is observed. A branch of the trigeminal nerve innervating the ventral aspect of the opercular area was sectioned, causing a dark patch which fades within 6 to 23 hours. Recutting the nerve distal to the first cut did not produce secondary darkening. (3) The most active melanophore pigment-aggregating agents, when injected subcutaneously, were epinephrine, norepinephrine, isopropyl norepinephrine, dopamine, melatonin and serotonin, with effective doses ranging from 0.02 to 0.10 micrograms. Three energizers, phenelzine, pheniprazine and etryptamine, were active at 0.3, 0.4 and 2.0 micrograms, respectively. Iproniazid and isocarboxazid were inactive. The metabolic products of epinephrine, metanephrine and mandelic acid, were inactive. Dihydroxyphenylalanine (DOPA) was also inactive. (4) Pretreatment with pyrogallol (5 mg. per kg.), an inhibitor of catechol-o-methyl transferase, resulted in marked potentiation of certain catechol amines. (5) Of the large number of drugs causing localized melanophore dispersion, the phenothiazine tranquilizers were most active, in an effective dose range of 0.08 to 5.0 micrograms. The most active members of this group were fluphenazine, perphenazine, fluorophenazine and thiopropazate. Pretreatment with pyrogallol (5 mg. per kg.) raised the effective dose of these agents by one or two orders of magnitude but had no influence on the other phenothiazines examined.

(trifluoperazine, prochlorperazine, acetophenazine, chlorpromazine, mepazine, proketazone). A similar response following pyrogallol was observed with Dibenamine and ergotamine. The more potent phenothiazines, therefore, appear to act as adrenergic blocking agents.

The extent of pigment aggregation or dispersion would presumably depend on the amount of endogenous catechol amine transmitter available to active sites on the melanophores. This would depend on the balance between neurosecretion by aggregating nerve fiber endings and physiological inactivation by COMT.

The research was supported by Grant MH 03903 from the National Institute of Mental Health to Oberlin College.

Swelling of the tubular system (TTS) in twitch fibers of Carcinus maenas. A. SELVERSTON, P. W. BRANDT AND J. P. REUBEN.

The closer carpopodite muscle contains fibers which in response to direct or indirect stimulation are almost exclusively phasic. At approximately 15 mv depolarization all-or-none action potentials and 80-msec twitches are produced. Electron microscopy revealed an extensive network of sarcolemmal invaginations. Tubules originating from these invaginations and from the surface penetrate into the fiber and form extensive diadic contacts with the sarcoplasmic reticulum at the A-I junctions. The crab fibers exhibit finite permeability to Cl, depolarizing transiently with a time constant of about 20 minutes when Cl is removed from the medium. As in gradedly responsive crayfish muscle fibers, the TTS of the crab twitch fibers can be caused to swell and vesiculate. Two procedures induced these changes. In one, fibers were loaded with KCl, then were replaced in a normal or a Cl-free Ringer. In the second, hyperpolarizing current pulses were passed through a KCl-filled intracellular microelectrode for 15 minutes to one hour. With both methods the swelling and subsequent vesiculations were visible with the light microscope. Swelling induced with currents was greatest under the electrode. Electron microscopy revealed that the swelling of the TTS was mainly in the tubules, but it could also be seen in the invaginations. Only the tubular components of the diads were swollen. Since Cl-efflux was the common condition to induce the morphological changes, the tubular membrane appears to be anion permselective. Two methods were used to estimate changes in membrane capacity when the fibers were vesiculated. Estimates based on the foot of the spike indicated no change in capacity, but cable analysis with square pulses indicated that the capacity was more than doubled when the tubules were swollen.

This work was supported by a Grass Foundation Fellowship to A. Selverston, by NIH grant (NB 05910-01A1) to P. W. Brandt, NIH grants (NB 03270-05 and NB 03728-04) to Dr. H. Grundfest. Dr. J. P. Reuben holds a career development award from NIH.

Visualization of radioactivity in Schistosoma mansoni by means of an image intensifier. ALFRED W. SENFT AND GEORGE T. REYNOLDS.

In an attempt to localize radioactivity in schistosome worms, specimens were made highly active by incubation in a maintenance medium to which had been added 1-proline- $U-C^{14}$. After 6 hours' incubation the worms were washed and placed on a Millipore filter for drying and counting. They were found to contain about 25,000 decays/worm/minute. The disc containing worms was then placed on a microscope slide and covered with 0.002"-thick Nuclear Enterprise scintillation plastic.

The slide was mounted at the focal point of a special Elgeet F 1.2 double lens focused so as to produce a 1:1 image on the cathode of the Image Intensifier. The tube used was an English Electric Valve type P829A, with tri-alkali cathode and a P-11 phosphor anode.

Faint incandescent trans-illumination was employed to focus the worms through the entire system, using the intensifier at low gain. After focusing, the specimen was viewed by means of the scintillation of the plastic cover slip. For this purpose the tube was driven at 28-36 kilovolts which provided an intensification of from 10^4 to 10^6 .

In some trials the worms were dusted with ZnS phosphor. This material has about 10 times the photo-emission per scintillation as compared to the plastic, but has the disadvantage of impairing ordinary microscopy.

Our results demonstrate that C^{14} -induced scintillation can be photographed by means of

Polaroid P-10,000 or P-3000 film. The resolution achieved has been extremely poor, which is a reflection of deficient optics and a noisy image intensifier. Improvement in optical efficiency above the presently calculated 1% level through development of a low-power objective having a numerical aperture greater than 0.30, would be most helpful. Concurrently, an image intensifier as quiet or better than those now used in astronomy will be required for optimal resolution.

However, we believe that it is technically possible to develop an instrument which will allow the virtual simultaneous microscopic observation of living or stained material and concomitant estimation of weak radioactivity contained therein.

Supported by NSF Grant GB-2673 and AEC Contract AT(30-1)-3406.

Effects of dopamine on Mercenaria mercenaria heart. MORRIS A. SPIRITES AND DAVID JACOBOWITZ.

The possibility that dopamine is a neurohumor in the heart of *M. mercenaria* has been explored. Treatment of the heart and visceral ganglia with paraformaldehyde, according to the method of Falck, resulted in the appearance of green-fluorescent, varicose nerve fibers. Because of a reported absence of other catecholamines in the nervous system of *M. mercenaria* by Sweeney, this fluorescence is probably due to dopamine. The latter was then tested on isolated and *in situ* heart preparations. The minimal effective dose of dopamine was 100 μg , which caused a negative inotropic and a positive chronotropic response. With large doses, a rapidly appearing increase in tonus was usually observed, as well as an occasional post-inhibitory positive inotropy and negative chronotropy. These effects could be almost completely blocked by a 5 μg /ml. dose of propranolol, a beta-adrenergic blocker. Dibenamine, an alpha-adrenergic blocker only partially inhibited dopamine effects. Methysergide (MLD), which blocks serotonin responses, had no effect on dopamine phenomena. These reactions were all confirmed using the *in-situ* prepared heart, except that the minimal effective dose was usually 1 μg . of dopamine. A rapidly appearing tachyphylaxis was noted, often within minutes, to all doses of dopamine in both types of preparations. Although this phenomenon is puzzling for a neurohumoral candidate, because of the probable presence of dopamine granules in the nerve fibers of the visceral ganglion and the heart and the low levels of the minimal effective doses, it is concluded that dopamine must be considered for such a role, at least for the heart.

In vivo determination of sodium turnover of tissues in the smooth dogfish, Mustelus canis. JOHN J. STANGEL AND W. T. W. POTTS.

The purpose of this preliminary investigation was to measure K, the fraction of total intracellular sodium exchanging with the blood per hour for several tissues of the smooth dogfish.

Thirty to 50 microcuries of Na^{24}Cl diluted to 0.5 ml. in isotopic NaCl were injected intravenously into the caudal vein of the smooth dogfish. The blood of several fish was sampled at various intervals and a log plot of counts per minute, cpm, was made. During the first 45–50 minutes the decline of activity could be represented by a single exponential term. The rate constant of decline was 0.78/hour.

Other dogfish were injected in a like manner, and killed at five minutes and at 30 minutes after isotope injection. Tissue samples were excised, weighed, and dissolved in hot nitric acid. Activities of these samples were measured as cpm/gram wet weight of tissue, and sodium was measured photometrically as μM Na/gram wet weight of tissue. Specific activities of tissues and blood were recorded and reported as the ratio of tissue specific activity to blood specific activity from the same animal. It was assumed that after five minutes the extracellular compartment was fully loaded, but the intracellular compartment was only slightly loaded. After 30 minutes the intracellular compartment was substantially loaded as well. From the specific activity of the tissues after five minutes and after 30 minutes both the size of the extracellular compartment and the rate constant for the intracellular compartment were computed with the aid of Moser and Emerson's equation (*J. Clin. Invest.* 1955).

The following are the preliminary constants found: kidney, 15/hour; rectal gland, 2.25/hour; muscle, 0.42/hour. These values seem to reflect the high rate of sodium extrusion by the

kidney and the rectal gland as compared to muscle, the greatest part of the body mass. The high activity of the kidney probably represents filtered sodium rather than intracellular sodium.

Sulphydryl balance in mitosis: The effect of mercaptoethanol on spindle birefringence. R. E. STEPHENS, SHINYA INOUÉ AND JOHN I. CLARK.

Rapkin, Sakai and others have shown cyclic changes in protein-bound sulphydryl groups during mitosis; Kawamura and Dan found such changes in the mitotic apparatus. Mazia and co-workers reported that mercaptoethanol and dithiodiglycol disrupt the structure of the mitotic apparatus prior to isolation. Experiments were carried out in order to test the hypothesis of Mazia that a balance between sulphydryl and disulfide groups is involved in the maintenance of the mitotic apparatus.

Application of mercaptoethanol at concentrations greater than 0.05 *M* caused complete disappearance of birefringence within two minutes in meiotic spindles of *Pectinaria gouldi* oöcytes; concentrations in the range of 0.01 to 0.05 *M* produced a rapid decrease in birefringence which thereafter remained constant. The birefringence was completely restored within five minutes upon perfusion with aerated sea water. Neither ethylene glycol nor ethanol caused any decrease in birefringence at similar solute concentrations. On the contrary, 0.1 *M* ethanol produced a marked increase.

Application of 0.01 *M* dithiodiglycol (oxidized mercaptoethanol) also caused a loss of birefringence which was irreversible with sea water perfusion. If the eggs were perfused with mercaptoethanol before the asters completely disappeared in dithiodiglycol, spindles re-formed upon sea water perfusion but were generally smaller than those prior to treatment.

Simultaneous application of dithiodiglycol and mercaptoethanol were antagonistic, causing a decrease in the rate of birefringence loss. Determination of exact molar ratios involved in such action was difficult, however, due to the questionable purity of commercial dithiodiglycol and to oxidation of dilute mercaptoethanol solutions.

Thus, formation of additional sulphydryl groups (through the action of mercaptoethanol) or disulfide linkages (through the action of dithiodiglycol) disrupt the ordered structure of the spindle. Dithiodiglycol action can be reversed only by subsequent reduction through mercaptoethanol. Mercaptoethanol and its oxidized form appear to act antagonistically. These findings are consistent with the theory that a change in balance between intramolecular sulphydryl and disulfide groups prevents association of spindle precursors.

Supported in part by National Cancer Institute Grant CA 10171, National Science Foundation Grant GB-5120, and National Institutes of Health Grant GM 14363.

Further studies on digenetic trematodes of the family Notocotylidae. HORACE W. STUNKARD.

Hydrobia salsa is a somewhat rare, brackish-water snail, described by Pilsbry (1905) as *Paludina salsa* from Cohasset, Massachusetts. During the summers of 1963, 1964, 1965 and 1966, over 5000 individuals have been examined for infection by larval trematodes, and five species of notocotylid cercariae have been recognized. These larvae emerge chiefly between 10 AM and 2 PM. They are ocellate, swim actively for a short time, the tail in advance, and accumulate on the light side of the container. In the course of one to three or four hours they encyst, often on the operculum or shell of the snail from which they emerged, but on any hard surface, including the wall of the container. The cysts are firmly attached by the hardening of the cystogenous material. Miriam Rothschild (1938) described three groups of notocotylid cercariae, the Yenchingensis Group, the Monostomi Group, and the Imbricata Group, based on differences in the structure of the excretory system. Feeding of cysts from individual snails to laboratory-reared eider and domestic ducklings has yielded five species of adult worms, belonging to three different genera. Two of the cercariae belong to the Yenchingensis Group, develop to maturity in the digestive ceca of the ducks, and are identified as *Notocotylus minutus* Stunkard, 1960 and an as yet undescribed species of *Notocotylus*. Two cercariae belong to the Monostomi Group, develop in the lumen of the intestine, and are identified as *Paramonostomum alveatum* (Mehlis in Creplin, 1846) and *Paramonostomum parvum* Stunkard and Dunihue, 1931. The fifth cercarial species belongs to the Imbricata Group,

develops in the bursa Fabricius, and may be identical with *Uniserialis gippyensis* Burton, 1938, from mallard ducks taken in England.

Chromatophore response in Loligo pealeii. . GEORGE SZABÓ AND AARON B. LERNER.

The chromatophore complex of the squid consists of a central pigment cell (chromatophore proper) and radial myoepithelial cells. We investigated the following problems: (1) Are muscle fibers present in the chromatophores which may be responsible for contraction of these cells? (2) Are the chromatophores themselves innervated? (3) How can the autonomous pulsation of the chromatophores of a decapitated animal be influenced by adrenergic or cholinergic drugs or by other compounds? (4) Has melatonin any effect on the chromatophores of cephalopods?

(1) Electron micrographs showed no evidence of muscle fibers inside the chromatophores. There are, however, abundant collagen fibers, arranged in bundles, around the chromatophores except where the smooth muscle cell or nerves are attached to the chromatophores. (2) There are nerve endings in close proximity to the cell membrane of the chromatophore, inserting themselves in between the muscle and the chromatophore. (3) Acetylcholine, atropine, tyramine, histamine, alpha and beta MSH, ACTH, melatonin, adrenaline and noradrenaline were injected subcutaneously into decapitated squids. The chromatophores contract immediately after decapitation. Several doses of 0.1 ml. solutions, in concentrations ranging from 1000 gamma to 0.1 gamma per ml. of sea water, were injected at one side of the animal and 0.1 ml. of sea water was injected on the contralateral side. In both control and experimental sites, stretching of skin due to the blister formation resulted in eventual expansion of chromatophores which took 1-3 minutes to develop. Only acetylcholine caused an immediate chromatophore expansion at concentrations 1000-100 gamma. It took progressively longer (10 seconds to 2 minutes) for the expansion to develop in lower concentrations. Atropine (1000 gamma per ml.) reversed the effect of acetylcholine, whereas the expansion of chromatophores caused by sea water was not reversed by atropine. Among the other substances tested only beta MSH and to a lesser degree alpha MSH showed some chromatophore-expansive action, which, however, was inconsistent. (4) When a subcutaneous blister is caused in a darkened, intact animal by injecting sea water under the skin, there is a slow contraction of chromatophores. Melatonin speeds up this blanching; within 30 seconds contraction occurs, whereas it takes 60-90 seconds for the sea water controls to develop any blanching.

Supported by a Research Grant CA 05401-07, USPHS, and Career Development Award USPHS Ke-GM-14,987 and CA 04679-07, USPHS.

Behavior and settling mechanism of planulae of Hydractinia echinata. MAE TEITELBAUM.

Experimental results on the behavior of the planulae of *Hydractinia echinata*, a colonial hydrozoan usually occurring on shells of *Pagurus longicarpus*, demonstrate that specific ecological factors influence the settling response. Planulae prefer to settle in pits and grooves and select the sculptured shell fragments of *Nassarius obsoleta* in preference to the smooth ones of *Polynices duplicatus* and *Littorina littorea*. Colonization often begins in the anal canal of a shell. Mechanical movement of shells or vibration of the dish increases settlement. The larvae prefer a clean glass surface to one covered with detrital film. Settlement increases as the density of larvae increases; 2-10% settlement is achieved with 100 larvae as opposed to 42-94% with 1000 larvae. The larvae are not attracted to adult colonies, i.e., they are not gregarious. Dishes with newly settled polyps appear to be favorable for metamorphosis, despite the fact that the individual polyps do not fuse to form one colony but form distinct colonies. In a half-light half-dark dish, the planulae prefer to settle in the light. Upon shading the light source, the planula straightens out, enabling it to hit an oncoming shell perpendicularly. The planulae have nematocysts all of the atrichous isorhiza type which are most densely distributed at the posterior end. Their function is believed to be that of adhesion. A larva will attach to a moving coverslip with its nematocysts or will hold on to a dish bottom by its tail despite suction of a pipette. The nematocysts do not fire upon addition of hermit crab extract, *Acartia* extract or electrical stimulus. They do fire upon application of dilute acid or a mechanical stimulus.

It is proposed that the larva attaches to a moving shell or crab leg by firing nematocysts and settles or may metamorphose after moving to a more protected spot.

Supported in part by Grant GM 535-05 from NIH to the MBL Marine Ecology Course, summer, 1965; by Grant GB-4509 from the NSF to the MBL Systematics-Ecology Program; and an NSF Fellowship for Teaching Assistants, summer 1966. The author gratefully acknowledges the helpful suggestions of Dr. D. Crisp during the summer of 1965.

The effects of reserpine and guanethidine sulfate on serotonin levels in Campanularia colonies. TAMARA M. THABES, CHARLES R. WYTTEBACH AND SANDRA E. COLLINS.

The capacity of reserpine and guanethidine sulfate to inhibit the tentacle closure response to tactile stimulation of *Campanularia* hydranths (Wytenbach, Thabes and Collins, 1966) suggested that we investigate the serotonin (5HT) content of this primitive hydroid and its alterations under treatment with these drugs.

Serotonin was extracted from whole colonies by the technique of Bogdanski *et al.* (1956), then complexed with ninhydrin according to the method of Snyder, Axelrod and Zweig (1965). Serotonin so extracted shows a single characteristic excitation peak at 390 m μ and a corresponding emission peak at about 480 m μ . Colonies were found to contain on the average 1.70 μ g. of serotonin per gram wet weight.

Bioassay, with the *Venus mercenaria* heart preparation, shows serotonin to be present in all parts of the colony, in the relative concentration (per unit protein) of gonangia) hydranths)) stems.

Colonies maintained in solutions of reserpine (1 or 10×10^{-6} gm./ml.) or guanethidine (2.5 or 200×10^{-6} gm./ml.) for periods of 30 minutes or 36 hours at 20° C. were assayed for serotonin per unit protein. In 5 cases of reserpine treatment, serotonin showed little change relative to the controls, averaging a decrease of 10%, with a range from -21% to +8%. Guanethidine treatment, among 7 cases, produced an average elevation of serotonin level to 20%, with a range from -27% to +73%. In two cases, in which a 30-minute recovery period in sea water had been allowed after 36-hour guanethidine treatment, a drop in 5HT back toward controls was noted.

Thus, reserpine treatment, of a dose and duration which produce measurable physiological effect, causes little depletion of serotonin. Physiologically active doses of guanethidine cause, unexpectedly, a variable but in most cases positive change in serotonin level. A clearcut relationship, therefore, between serotonin depletion and impaired neural function does not exist.

Supported by NSF grant number GB-2663.

A study of the effects of divalent cations on squid giant axons. A. WATANABE, I. TASAKI AND L. LERMAN.

After enzymatic removal of axoplasm, and under intracellular perfusion with 25 to 100 mM CsF, the squid giant axon maintained excitability in media free of univalent cation salts. In external media containing Ca, Ba, or Sr as the sole cation species, the action potential was typically of long duration (0.1 to 20 seconds) and between 70 to 100 mV in amplitude. Action potential duration decreased as the calcium concentration was increased in a range between 50 and 400 mM. With excitation, membrane resistance fell sharply, reaching a level of approximately one-sixth its resting value at the peak of the action potential. Total elimination of divalent cation with EDTA from external medium containing 600 mM NaCl resulted in a rapid loss of excitability. Subsequently recovery occurred with addition of divalent cation.

"Abolition" of action potentials by a brief inward current pulse was demonstrable. When the duration of an action potential was long, an inward directed current pulse applied during the plateau of the action potential resulted in a "all-or-none," premature termination of the action potential.

In external media containing 400 mM NaCl and 50 mM Ca⁴⁵Cl₂, the calcium influx during resting and excited states was measured. Average calcium influx increased by a factor of approximately six during suprathreshold biphasic stimulation at 50 shocks/second. Rapid subthreshold stimulation showed no observable effect on Ca influx.

Cytological studies on the inhibition of early cleavage by estradiol 17 β in Arbacia punctulata. D. G. WHITTINGHAM AND C. R. AUSTIN.

Early cleavage stages in *Arbacia* were inhibited with estradiol 17 β (Agrell, 1954) at concentrations of 10^{-4} M and 10^{-5} M in sea water.

It has now been found that treatment of unfertilized eggs for 5, 10, and 15 minutes did not prevent cleavage and normal development if they were returned to normal sea water prior to semination. However, if eggs remained in estradiol solution they failed to divide at the first cleavage division although sperm entry and elevation of the fertilization membrane were unimpeded. Pronuclear fusion failed to take place (60–70% of eggs) if unfertilized eggs were placed in estradiol 10 and 15 minutes prior to semination; the chromosomes appeared at metaphase in two separate haploid plates. Pronuclear fusion may have been prevented by failure of aster formation normally responsible for bringing the pronuclei together.

Unfertilized eggs placed in estradiol 5 minutes before semination and eggs placed in it after semination revealed no impairment of pronuclear fusion and aster formation, but spindle formation was not well defined and chromosomes appeared scattered at the metaphase stage.

Prolonged treatment beyond the metaphase stage (30–40 minutes) led to the development of tripolar, tetrapolar and more complex spindles (40–50% of the eggs had tetrapolar spindles). Eventually multinucleate cells were formed which sometimes fragmented, yielding "blastomeres" of unequal size containing one, several or no nuclei. Formation of tetrapolar spindles at the time of the second metaphase suggests that centriolar replication takes place, with the production of a second spindle. Although the steroid caused a delay at metaphase, it apparently did not prevent further development of the nuclear cycle taking place. Essentially cytokinesis was delayed, fragmentation of the eggs occurring 80–90 minutes after semination.

The primary effect of estradiol thus appears to be upon spindle structure, interfering with division and causing detachment of chromosomes. Centriolar replication and the nuclear cycle, including chromosome replication, evidently proceed unimpaired.

Facilities made available through training grant 5 T1 HD 26-05 from the National Institutes of Health.

The physiological effects of reserpine and guanethidine sulfate on Campanularia hydranths. CHARLES R. WYTTEBACH, TAMARA M. THABES AND SANDRA E. COLLINS.

In order to gain insight into the nature of nerve transmission in coelenterates, we have studied the effects of two serotonin- and catecholamine-depleting drugs upon the response of *Campanularia flexuosa* hydranths to both tactile and chemical stimulation.

Using contraction of the entire tentacle ring upon mechanical stimulation of just 3 to 5 tentacles as a criterion for the ability to receive and produce an integrated response to tactile stimulation, guanethidine produced a very clearcut dose-effect curve. Average non-responsiveness at the extreme concentrations tested, 1.56×10^{-6} and 5×10^{-4} gm./ml., were 8.9% and 97.5%, respectively, among nearly 200 hydranths tested, relative to a control value of 6.7%. At all eight doses tested, maximal or near maximal effect was noted after just 5 minutes' exposure. Recovery is equally rapid: non-responsiveness in the colony held at 5×10^{-4} gm./ml. for 90 hours dropped, after return to normal sea water, to 35%, 25% and 15% at 5 minutes, 90 minutes and 10 hours, respectively.

Dose-response data for reserpine are incomplete due to its limited solubility in sea water and because considerable fluctuation in response throughout the observation period made it unfeasible to determine a lower limit of effective concentration. However, at saturation (about 1×10^{-5} gm./ml.) and at 1×10^{-6} gm./ml., reserpine produced an average non-responsiveness over the 5-day observation period of respectively, 45% and 35% among nearly 500 hydranths tested, relative to a control figure of 12%. In contrast to guanethidine, the reserpine effect is not seen until after three hours exposure, and recovery after prolonged treatment is much slower.

Regardless of dose or time in either drug, all hydranths tested reacted positively to the chemical stimulus employed, i.e., they showed a normal feeding response on exposure to a drop of supernatant from a centrifuged *Artemia* homogenate.

The differential effect of these drugs in modifying response to tactile and chemical stimulation suggests that the neural pathways involved are pharmacologically distinct.

Supported by NSF grant number GB-2663.

Protein synthesis in dogfish cornea epithelial cells. S. ZIGMAN, S. LERMAN, S. ROCKFORD AND J. TUTTLE.

Dogfish (*Mustelus canis*) cornea epithelial cells are able to incorporate H^3 -uridine into RNA and C^{14} -amino acids into protein when incubated at 20° C. in elasmobranch Ringer's solution with 95% O_2 :5% CO_2 . The amount of incorporation into insoluble (nuclear), ribosomal, and soluble RNA and protein increases from a low level in 15 minutes to a plateau at 6 hours of incubation.

When fresh cells are homogenized and the 1400 g residue removed, 5% to 20% sucrose density centrifugation yielded an ultraviolet (UV) absorption (260 m μ) profile with a peak due to heavy particles in tube 7 and lighter particles in tube 27. Electron microscopy of solutions taken from these tubes showed polyribosomes (clusters of ribosomes attached by RNA strands) present in tube 7 and single ribosomes present in tube 27. Gentle ribonuclease treatment resulted in a loss of the polyribosome peak due to RNA chain breakage.

When actinomycin D (40 μ g./ml.) was added to 3-hour incubations, a marked depression of polyribosomal RNA and protein synthesis was found. Differential centrifugation of cell homogenates indicated a 4- to 5-fold inhibition of ribosomal RNA and protein synthesis. RNA and protein synthesis in the insoluble and soluble fractions was inhibited 2- to 3-fold.

Two hours of UV irradiation led to a less marked depression of polyribosomal RNA and protein synthesis, as shown by gradient centrifugation. This depression was limited to ribosomal and soluble fractions. Control to irradiated ratios were approximately 2.

The results show that the epithelial cells of the dogfish cornea can synthesize protein on polyribosomes during incubation in simple media, and that protein and RNA syntheses are inhibited by actinomycin D and UV light.

Supported by Fight For Sight Grant G325 and Student Fellowship SF313 of the National Council To Combat Blindness.

A new method for the extraction of living Thalassopsammon from intertidal and subtidal marine sands. DONALD J. ZINN.

A gentle, efficient and repeatable method, superior to manual and mechanical stirring and shaking in not damaging or destroying protozoa and contractile metazoa, has been developed. It is sturdier, quicker and more portable than the Boisseau Tubes, and it is faster and more readily usable for sands with heavy lacunar detritus than the Uhlig Sea-ice System. It extracts 70% to 80% of the total psammon population from 100-cc. sand samples.

The central instrument used in this method extracts Annelida, Mollusca, Nematoda, Arthropoda, Nemertinea and Foraminifera best, and is less successful in varying degree in removing Protozoa, Tardigrada, Gastrotricha, Ostracoda and Turbellaria. The removal of organisms that adhere tenaciously to sand grains can be facilitated by treating the sample for about 10 minutes with 6% magnesium chloride added to filtered sea water.

In practice, a 100-cc. sample of sand is placed in a vertical, transparent plastic tube 10" long with an inside diameter 1 $\frac{1}{8}$ " and plugged with a No. 8 rubber stopper at the bottom. Depending on the dryness of the sand, 50 to 100 cc. of filtered sea water are added at the top. Air is then bubbled through the sand from a small piston-type aquarium pump connected to a plastic "Y" tube that leads to two 12" lengths of rigid plastic tubing ($\frac{3}{8}$ " inside diameter) cemented on opposite sides of the larger tube with their lower ends $\frac{3}{8}$ " from the rubber stopper. After 5 minutes bubbling, and with the pump still engaged, the tube is released from the clamp holding it vertically to a ring stand, and the water with the *Thalassopsammon* is poured into a container. It is gently mixed, poured in aliquots into small plastic petri dishes, permitted to settle, and then placed on the dissecting microscope stage for counting and sorting.

Supported by Grant GB-4116 from the National Science Foundation, and Grant GB-4509 from the National Science Foundation to the Marine Biological Laboratory, Systematics-Ecology Program, Woods Hole, Massachusetts.

THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE ROLE OF SODIUM CHLORIDE IN SEQUENTIAL INDUCTION OF THE PRESUMPTIVE EPIDERMIS OF *RANA* *PIPIENS* GASTRULAE¹

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Previous investigations have shown that ions will induce various cell types from the presumptive epidermis of the *Rana pipiens* gastrula (Barth and Barth, 1959, 1962, 1963, 1964, 1966). When it was found that sucrose also would induce (Barth, 1965), the question arose as to whether sucrose acted directly as an inductor or indirectly by facilitating the penetration of ions. A study of the effects of sucrose in relation to various concentrations of sodium chloride was undertaken.

METHODS

The solutions and procedures used for operation, treatment, and culture of small aggregates of cells from the *Rana pipiens* embryo have been described in detail (Barth and Barth, 1959, 1962, 1963, 1964, 1966). Essentially the procedure consists of the following steps: (1) Presumptive epidermis regions, for example, are dissected out in standard solution and treated briefly with Versene (EDTA) to loosen the pigment coat layer from underlying presumptive epidermis cells; (2) aggregates consisting of approximately 100 cells each are teased out from the presumptive epidermis and allowed to heal for 10–15 minutes before transfer to treatment or culture solutions; (3) cultures of such aggregates in small glass stender dishes prepared and maintained under sterile conditions are able to be observed daily in the living condition.

RESULTS

Table I records the data obtained with sucrose substituted for the sodium chloride in the standard solution used for culture of the presumptive epidermis. Low concentrations of sucrose (exps. 1 and 2) induce radial nerve and slate gray epithelium, while higher concentrations (exps. 7, 8 and 9) induce pigment cells and nerve. The higher concentrations applied for different periods of time induce first nerve, then slate gray epithelium and finally pigment cells (exp. 11). Thus,

¹ This work was supported by a grant from the Department of Health, Education, and Welfare, HD 00701-02, to the Marine Biological Laboratory, Woods Hole, Massachusetts.

TABLE I

Sequential induction by solutions in which NaCl is replaced by sucrose

NaCl is omitted from the medium and varying amounts of sucrose added in its place.

In this and succeeding tables the headings are to be interpreted as follows:

Stage no.: Shumway (1940); conc.: concentration of substances in milligrams per milliliter of solution; hrs.: time in hours during which aggregates are exposed to the substances indicated; types of cellular differentiation: as in Barth and Barth (1962, 1963, 1964); UPS = unipolar spongioblasts.

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11	29.0	6.0	25	Radial nerve
	11	29.0	8.5	42	Radial nerve
2	11	43.0	3.3	75	Radial nerve, epithelium
	11	43.0	6.3	75	Nerve, slate gray epithelium, epithelium
3	11 —	43.0	4.0	40	Nerve
	11 —	43.0	5.5	35	Nerve, pigment cells
4	11 +	58.0	2.5	35	Nerve, rare pigment cells
5	11	58.0	4.5	75	Nerve, pigment cells
6	11	58.0	5.0	75	Nerve, pigment cells
7	11 —	58.0	5.0	70	Nerve, pigment cells
8	11 —	58.0	6.0	75	Nerve, pigment cells
9	11	58.0	9.5	50	Pigment cells, slate gray epithelium, nerve
10	11	58.0	5.0	75	Nerve, slate gray epithelium, UPS
	11	58.0	10.5	75	Pigment cells, nerve
11	11	58.0	3.0	25	Nerve, UPS
	11	58.0	6.0	50	Nerve, slate gray epithelium, UPS
	11	58.0	8.5	75	Pigment cells, nerve
12	11	65.0	4.5	75	Nerve, pigment cells
13	11 —	65.0	6.0	75	Nerve, pigment cells
14	11 —	87.0	4.0	60	Nerve
15	11 —	116.0	3.0	28	Nerve
	11 —	116.0	4.0	32	Dead

sucrose appears to induce sequentially a variety of cell types as do various ions (Barth, 1965).

Since the above experiments were done with a medium containing the normal concentrations of K^+ , Ca^{++} , Mg^{++} , HCO_3^- and phosphate ions, any of these ions

might have acted as the inductor. Therefore sucrose was applied to the cells in the complete absence of ions.

Table II records the results of experiments in which various concentrations of sucrose dissolved in glass-distilled water were applied to presumptive epidermis for varying lengths of time. Experiment 1 shows that a low concentration of sucrose induces nerve and slate gray epithelium. Higher concentrations (exps. 3, 4 and 7) induce pigment cells and nerve. Experiments 2 and 6 show sequential induction of radial nerve, spreading nerve and unipolar spongioblasts, while in experiment 3 with longer times of exposure pigment cells are induced. Thus, sucrose in the complete absence of ions will induce the various cell types which are also induced by ions.

TABLE II
Sequential induction by sucrose in glass-distilled water

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11 —	43.0	5.0	28	Nerve, slate gray epithelium
2	11	58.0	0.5	20	Ciliated epithelium
	11	58.0	0.75	20	Radial nerve, ciliated epithelium
	11	58.0	1.0	20	Spreading nerve
	11	58.0	1.5	20	Spreading nerve, UPS
3	11	58.0	1.0	25	Nerve, pigment cells
	11	58.0	2.0	25	Pigment cells, nerve
	11	58.0	2.5	25	Pigment cells, nerve, dead cells
4	11 +	58.0	2.5	28	Pigment cells, nerve
5	11 —	58.0	0.75	40	Spreading nerve, UPS
6	11 —	58.0	0.1	35	Ciliated masses
	11 —	58.0	0.5	40	Nerve, ciliated epithelium
	11 —	58.0	1.25	35	Nerve, ciliated epithelium
	11 —	58.0	1.75	40	Nerve, UPS
7	12 —	58.0	0.4	40	Spreading nerve, radial nerve
	12 —	58.0	1.5	36	Nerve, slate gray epithelium, pigment cells
8	11	68.0	1.0	40	Spreading nerve, slate gray epithelium, pigment cells
	11	68.0	1.7	35	Spreading nerve, pigment cells
9	11	90.0	1.0		Spreading nerve, slate gray epithelium
	11	90.0	1.7		Spreading nerve
10	11	137.0	1.0		Spreading nerve, radial nerve
	11	137.0	1.5		Spreading nerve, UPS
	11	137.0	2.0		Spreading nerve, UPS
	11	137.0	3.0		Dead cells

TABLE III

Induction by sugars other than sucrose, dissolved in glass-distilled water
 gl = glucose; lac = lactose; xyl = xylose.

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11	lac 58	4.5	35	Pigment cells
	11	lac 58	5.1	40	Pigment cells
2	11 —	lac 58	4.0	28	Pigment cells
	11 —	lac 58	4.5	32	Pigment cells
3	11 —	gl 31	2.0	75	Spreading nerve, UPS
	11 —	gl 31	3.0	75	Spreading nerve, UPS, slate gray epithelium
4	11 —	gl 31	4.0	32	Pigment cells
	11 —	gl 31	4.5	20	Pigment cells
5	11	gl 31	4.5	40	Pigment cells
	11	gl 31	5.1	25	Pigment cells
6	11	xyl 20	0.5		Nerve, ciliated masses
	11	xyl 20	1.0		Spreading nerve
	11	xyl 20	2.0		Pigment cells, slate gray epithelium

The next question asked was: Is sucrose peculiar in some respect as regards induction, or will other sugars induce? Table III records the results of experiments with other sugars when these compounds are dissolved in glass-distilled water. Experiments 1 and 2 show that lactose will induce pigment cells, while experiments 3, 4 and 5 demonstrate the sequential action of glucose when applied for different intervals of time. Experiment 6 shows sequential induction by xylose with respect to time. Thus sucrose is not unique in its ability to induce but shares this quality with lactose, glucose and xylose.

Speculations on the nature of the action of sugars as inductors led to a number of possible actions. First of all the sugars could be acting directly as inductors by some unknown mechanism or they could act indirectly by altering the cells in some manner so that subsequently the ions of the standard solution would induce. The possibility that the ions of the standard solution might induce is indicated by the fact that in some species of Amphibia, namely *Ambystoma maculatum* and *Ambystoma opacum*, presumptive epidermis will differentiate into brain without the presence of extraneous inductors (Barth, 1941; Holtfreter, 1944). Therefore the standard salt solution must have been the inductor. We also had some evidence that some substances would induce if applied for a short time, while long periods of treatment resulted in ciliated epidermis. Such a finding would be consistent with the hypothesis that induction occurred in the standard salt solution and not in the substance tested. This follows from the known fact that ability of cells to be induced by an inductor disappears with time.

Experiments therefore were set up to test the hypothesis that sugars did not

actually induce but that the induction occurred when the cells were transferred to the standard salt solution.

Two types of experiments were designed to test the idea. In one, the cells were to be kept in sucrose until the period of competence was concluded and then returned to the standard solution. Under these conditions no induction was to be expected. The other type of experiment consisted in reducing the ionic strength of the standard solution in an attempt to reduce its inductive capacity. The first type of experiment proved to be too difficult to carry out, as the cells would not survive sucrose treatment in absence of ions when exposed during the entire period of competence. The second type of experiment gave definite results.

Table IV records the results of experiments in which the ionic strength of the standard salt solution was reduced by varying the concentration of sodium chloride. The standard solution contains 5.15 mg. NaCl/ml. Experiment 1 shows that a concentration of 1.28 mg./ml. is too low for continuous treatment of the cells, although ciliated masses will develop after 7.5 hours treatment. Experiments 2 and 3 show that 2.0 mg./ml. is the minimum concentration of sodium chloride which may be used for continuous culture of the cells. Experiments 4-8 record the results with 2.55, 2.57 and 3.0 mg./ml. of sodium chloride.

The first results of the effect of a reduction in ionic strength upon induction are shown in Table V, experiments 1, 2 and 3. There was no induction by sucrose

TABLE IV

The effect of various concentrations of sodium chloride

Normal concentration is 5.15 mg./ml. Other ions are present in normal concentration.

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11	1.28	1.5	25	Radial nerve, spreading nerve, little ciliated epithelium
	11	1.28	7.5	25	Ciliated masses, many unattached single cells
	11	1.28	cont.	25	Dead cells
2	11	2.00	7.5	30	Masses with voluminous mucus, some cilia
	11	2.00	cont.	20	Ciliated masses with voluminous mucus
3	11—	2.00	6.0	25	Ciliated masses with some mucus
	11—	2.00	cont.	20	Ciliated masses with voluminous mucus
4	11	2.55	3.0	25	Ciliated epithelium
	11	2.55	6.0	25	Ciliated epithelium
	11	2.55	8.5	25	Ciliated epithelium
5	11	2.57	6.0	25	Ciliated masses with some mucus
	11	2.57	cont.	25	Ciliated masses, ciliated epithelium
6	11	2.57	cont.	25	Ciliated masses
7	11	2.57	cont.	25	Ciliated masses with a little mucus, ciliated epithelium
8	11—	3.00	cont.	25	Ciliated epithelium

TABLE V

Lack of induction by sucrose when followed by culture in low concentrations of sodium chloride

Sucrose dissolved in glass-distilled water. Standard salt solution contains 5.15 mg. NaCl/ml. Other ions are present in same concentrations as in our standard solution.

Exp. no.	Stage no.	Sucrose treatment		No. of aggregates	NaCl conc. in culture	Types of cellular differentiation
		Conc.	Hrs.			
1	11 —	58.0	1.25	35	5.15	Spreading nerve, UPS
	11 —	58.0	1.25	40	2.00	Ciliated masses, mucus
2	11 —	58.0	2.0	35	5.15	Spreading nerve, UPS
	11 —	58.0	2.0	40	2.0	Ciliated masses
3	11 —	58.0	1.0	38	2.0	Ciliated masses
	11 —	58.0	1.5	40	2.0	Ciliated masses
	11 —	58.0	2.0	35	2.0	Ciliated masses
	11 —	58.0	2.3	35	2.0	Ciliated masses
4	11	58.0	2.0	25	2.57	Ciliated masses
	11	58.0	2.5	25	2.57	Ciliated masses, mucus
	11	58.0	3.0	25	2.57	Ciliated masses, mucus
5	11	58.0	2.0	40	2.57	Ciliated masses
6	11	58.0	2.0	40	2.57	Ciliated masses, little mucus, ciliated epithelium
7	11 —	58.0	2.0	40	2.57	Ciliated masses
8	11	58.0	2.0	35	2.57	Ciliated masses
9	11	58.0	2.3	25	2.57	Ciliated masses, mucus, ciliated epithelium
10	11	58.0	2.2	40	2.57	Ciliated masses, ciliated epithelium
11	11	58.0	2.8	25	2.57	Ciliated masses, mucus, ciliated epithelium
12	11	58.0	2.3	25	2.57	Ciliated masses, mucus, ciliated epithelium
13	11	58.0	1.8	40	3.00	Spreading nerve, radial nerve
14	11 —	59.0	2.3	40	2.57	Ciliated epithelium
	11 —	59.0	2.3	40	3.50	Spreading nerve
	11 —	59.0	2.8	40	2.57	Ciliated epithelium, rare nerve
	11 —	59.0	2.8	40	3.50	Spreading nerve, UPS
15	11 —	58.0	4.0	25	3.75	Spreading nerve
16	11	58.0	2.7	35	4.00	Spreading nerve, pigment cells

when the cells were returned to 2.0 mg./ml., but good induction when they were returned to 5.15 mg./ml. Experiments 4 through 12 resulted in no induction with sucrose when the cells were returned to sodium chloride at a concentration of 2.57

mg./ml. If, after sucrose treatment, the cells were returned to a solution containing 3.0 mg./ml., induction of radial nerve and spreading nerve took place. Experiment 14 shows that induction occurs in a solution containing 3.50 mg. of sodium chloride per milliliter but not at a concentration of 2.57 mg./ml. At concentrations of 3.75 and 4.0 mg./ml. induction also occurs (exps. 15 and 16).

It is clear that induction with sucrose is dependent upon the concentration of sodium chloride in the solution into which the cells are subsequently transferred. Table VI records the data from experiments in which after treatment with sucrose the cells were transferred to different concentrations of sodium chloride. The extent of induction is proportional to the concentration of sodium chloride.

Either the cells are not induced by sucrose or the cells are induced by sucrose but cannot differentiate in low concentrations of sodium chloride. Table VII demonstrates that induced cells do differentiate in low concentrations of sodium chloride. In these experiments the cells are first treated with sucrose, then transferred to high concentrations of sodium chloride for varying periods of time, and lastly transferred to low sodium chloride for culture. Experiment 1 shows that while a two-hour post-treatment with high sodium chloride (4.25 mg./ml.) results mostly in ciliated cells with a little radial nerve present, a five-hour post-treatment

TABLE VI

After treatment with sucrose, induction is proportional to the concentration of NaCl in the culture medium

Sucrose dissolved in glass-distilled water. Concentration of NaCl is varied, but other ions are as in our standard solution.

Exp. no.	Stage no.	Sucrose treatment		No. of aggregates	NaCl conc. in culture	Types of cellular differentiation
		Conc.	Hrs.			
1	11 —	58.0	2.0	35	2.57	Ciliated masses
	11 —	58.0	2.0	40	3.00	Ciliated masses, radial nerve, spreading nerve
	11 —	58.0	2.0	35	3.50	Spreading nerve, radial nerve
	11 —	58.0	2.0	40	4.00	Spreading nerve, UPS
2	11 —	58.0	2.1	35	2.57	Ciliated masses, mucus
	11 —	58.0	2.1	40	3.00	Ciliated masses, spreading nerve
	11 —	58.0	2.1	40	4.00	Spreading nerve, UPS, slate gray epithelium, pigment cells
3	11 —	58.0	2.5	30	2.00	All ciliated masses
	11 —	58.0	2.5	30	2.57	Ciliated masses, rare nerve
	11 —	58.0	2.5	30	3.00	Ciliated masses, ciliated epithelium, nerve
	11 —	58.0	2.5	30	3.75	Nerve, no ciliated cells
	11 —	58.0	2.5	30	4.50	Spreading nerve, UPS
4	11	58.0	2.1	25	2.25	Ciliated masses, mucus
	11	58.0	2.1	25	2.57	Ciliated masses
	11	58.0	2.1	25	3.00	Nerve, cilia rare
	11	58.0	2.1	25	3.50	Spreading nerve, UPS
	11	58.0	2.1	25	3.50	UPS, spreading nerve

TABLE VII

After pre-treatment with sucrose, induction is proportional to the time of exposure to high concentrations of NaCl

Aggregates are pre-treated with sucrose in glass-distilled water, then transferred to a "high" concentration of NaCl for varying lengths of time (post-treatment), and finally transferred to a culture medium of "low" NaCl content. "High" concentration is from 4.25 to 5.15 mg. of NaCl per ml. of solution containing the other ions in normal concentrations.

Exp. no.	Stage no.	Sucrose pre-treatment		No. of aggregates	NaCl post-treatment		NaCl conc. in culture	Types of cellular differentiation
		Conc.	Hrs.		Conc.	Hrs.		
1	11 —	58.0	2.0	35	4.25	2.0	2.57	Ciliated masses, ciliated epithelium, radial nerve
	11 —	58.0	2.0	40	4.25	5.0	2.57	Spreading nerve, no cilia
	11 —	58.0	2.0	40	4.25	7.0	2.57	Spreading nerve, no cilia
	11 —	58.0	2.0	35	4.25	19.0	2.57	Spreading nerve, no cilia
2	11	58.0	2.3	25	—	—	2.25	Ciliated masses
	11	58.0	2.3	25	4.25	1.0	2.25	Ciliated masses
	11	58.0	2.3	25	4.25	2.0	2.25	Nerve, ciliated masses
	11	58.0	2.3	25	4.25	5.0	2.25	Nerve, no cilia
	11	58.0	2.3	25	4.25	19.0	2.25	Nerve, no cilia
3	11 —	54.0	2.0	35	5.15	0.2	2.57	Ciliated masses, rare nerve
	11 —	54.0	2.0	40	5.15	2.0	2.57	Spreading nerve, no cilia
	11 —	54.0	2.0	40	5.15	4.5	2.57	Extensive spreading nerve, no cilia
	11 —	54.0	2.0	40	5.15	19.5	2.57	Extensive spreading nerve, no cilia
4	11 —	58.0	2.0	40	5.15	19.0	2.57	Nerve, no cilia
5	11 —	58.0	2.0	50	5.15	19.0	2.57	Nerve, no cilia

induces spreading nerve. In experiment 3 where post-treatment consisted of 5.15 mg. of sodium chloride per milliliter of solution, induction occurred in two hours. The extent of induction is proportional to the time of post-treatment with high concentrations of sodium chloride.

It is clear that sucrose will not induce unless followed by a treatment with a solution containing from 3.0 to 5.15 mg. of sodium chloride. Do sucrose and sodium chloride have similar effects so that they are synergetic or does sucrose merely prepare the cells for induction by sodium chloride? Table VIII records the results of experiments designed to answer this question. Experiment 1 shows that 29.0 mg. of sucrose will induce nerve while 2.57 mg. of sodium chloride has no inductive properties. The two compounds in combination have no inductive properties. Sodium chloride, therefore, applied simultaneously with sucrose antagonizes the inductive action of sucrose and the cells differentiate into ciliated masses instead of nerve.

Experiment 2 shows that 29.0 mg. of sucrose applied for 8 hours will induce as far as pigment cells, but when combined with sodium chloride the sucrose has no inductive ability. Experiments 3, 4, 5 and 6 confirm and extend the results of exps. 1 and 2.

Therefore sucrose cannot induce by itself unless followed by high sodium chloride, nor can sucrose in combination with sodium chloride induce regardless of subsequent treatment. It may be concluded, therefore, that the action of sucrose in the absence of sodium chloride is to alter the cell surfaces so as to permit sodium chloride and other ions to penetrate.

Actually the alteration of the cell surfaces is probably due to *lack* of sodium chloride in the solution and not to the presence of sucrose. Sucrose probably merely maintains the osmotic pressure necessary for survival of cells while lack of sodium chloride produces the alteration in the cell surfaces. The fact that

TABLE VIII

The antagonism of sucrose and NaCl when applied together

In these experiments the concentrations of all ions except Na^+ and Cl^- are kept constant. Various concentrations of NaCl (Na) and/or sucrose (S) are added to a standard solution lacking NaCl. Culture of the aggregates after treatment is also in the presence of the normal concentrations of all ions except Na^+ and Cl^- .

Exp. no.	Stage no.	Treatment concentrations	Hrs.	No. of aggregates	Culture	Types of cellular differentiation
1	11	29 S	6.0	25	5.15 Na	Nerve
	11	29 S	6.0	25	2.57 Na	Nerve, ciliated masses
	11	2.57 Na	6.0	25	2.57 Na	Ciliated masses, some mucus
	11	2.57 Na	6.0	25	5.15 Na	Ciliated masses, ciliated epithelium
	11	29 S + 2.57 Na	6.0	25	2.57 Na	Ciliated masses, some mucus
	11	29 S + 2.57 Na	6.0	25	5.15 Na	Ciliated epithelium
2	11 —	29 S	3.0	20	5.15 Na	Nerve
	11 —	29 S	3.0	20	2.57 Na	Nerve
	11 —	29 S + 2.57 Na	3.0	20	5.15 Na	Ciliated epithelium
	11 —	29 S + 2.57 Na	3.0	20	2.57 Na	Ciliated masses
	11 —	29 S	8.0	20	5.15 Na	Nerve
	11 —	29 S	8.0	20	2.57 Na	Pigment cells, nerve
	11 —	29 S + 2.57 Na	8.0	20	5.15 Na	Ciliated epithelium, radial nerve
3	11	29 S + 2.57 Na	5.3	25	5.15 Na	Ciliated masses
	11	29 S + 2.57 Na	5.3	25	29 S + 2.57 Na	Ciliated masses
4	11	29 S	6.0	25	5.15 Na	Radial nerve, spreading nerve, UPS
	11	29 S	8.0	40	5.15 Na	Radial nerve, spreading nerve, UPS
	11	29 S	3.0	25	5.15 Na	Radial nerve
	11	2.55 Na	3.0	25	5.15 Na	Ciliated epithelium
	11	2.55 Na	6.0	25	5.15 Na	Ciliated epithelium
	11	2.55 Na	8.0	25	5.15 Na	Ciliated epithelium
5	11	29 S + 2.55 Na	9.5	50	5.15 Na	Ciliated epithelium
	11	5.15 Na	9.5	50	5.15 Na	Ciliated epithelium
	11	58 S	9.5	50	5.15 Na	Pigment cells, nerve, slate gray epithelium
6	11	34 S + 5.15 Na	2.0	35	5.15 Na	Ciliated masses
	11	34 S + 5.15 Na	5.5	40	5.15 Na	Ciliated masses
	11	34 S + 5.15 Na	18.0	75	5.15 Na	Ciliated masses

TABLE IX

Correlation between pre-treatment with solutions lacking sodium chloride and induction by standard salt solution

Sodium chloride is omitted from the standard salt solution and other substances are added with or without sodium chloride. After a period of treatment the aggregates are transferred to standard solution containing 5.15 mg. NaCl/ml. E.G. = ethylene glycol; gl = glycine; suc = sucrose.

Exp. no.	Stage	Treatment concentrations	hrs.	No. of aggregates	Types of cellular differentiation
1	11	13 gl	0.5	30	Spreading nerve, ciliated masses
	11	13 gl	1.0	30	Spreading nerve
	11	13 gl	1.7	30	Dead cells, spreading nerve
	11	13 gl	3.0	10	Dead cells
2	11	31 E.G. + 2.0 NaCl	1.0	25	Radial nerve, spreading nerve, epithelium
	11	31 E.G. + 2.0 NaCl	2.0	25	Epithelium, ciliated masses, mucus
	11	31 E.G. + 2.0 NaCl	4.0	25	Ciliated masses, voluminous mucus
	11	31 E.G. + 5.15 NaCl	0.3	25	Ciliated masses, mucus, epithelium, nerve
	11	31 E.G. + 5.15 NaCl	5.0	25	Ciliated masses, mucus
	11	31 E.G.	1.0	25	Dead cells
	11	31 E.G.	2.0	25	Dead cells
	11	6 E.G. + 5.15 NaCl	1.5	30	Ciliated masses, mucus
	11	6 E.G. + 5.15 NaCl	6.0	35	Ciliated masses, mucus
	11	6 E.G. + 5.15 NaCl	16.0	25	Ciliated masses, mucus
3	11	0.0 NaCl	0.25	35	Ciliated masses, epithelium, rare nerve
	11	0.0 NaCl	0.5	20	Nerve, epithelium, rare UPS
	11	0.0 NaCl	0.75	12	Spreading nerve
	11	0.0 NaCl	3.0	50	Cytolyzed
	11	58 suc	0.75	40	Spreading nerve, UPS
	11	0.65 NaCl	0.75	25	Spreading nerve, epithelium
	11	0.65 NaCl	1.0	25	Nerve, epithelium

sodium chloride added to sucrose results in no induction suggests strongly that the induction is by means of a lack of sodium chloride.

Additional evidence that the lack of sodium chloride so alters the cell surfaces that subsequent exposure to high concentrations of sodium chloride results in induction comes from a few experiments recorded in Table IX. Experiment 1 shows the effect of substitution of glycine for sodium chloride. Short exposures to this solution result in the induction of spreading nerve after the aggregates are returned to high concentrations of sodium chloride. When ethylene glycol is substituted for sodium chloride the cells do not survive, but when ethylene glycol is combined with a low concentration of sodium chloride induction of nerve occurs when the cells are returned to a high concentration of sodium chloride (exp. 2). When ethylene glycol is added to a high concentration of sodium chloride there is no inductive activity of the solution. Thus, again the alteration of cell surfaces

appears to be the result of low sodium chloride content rather than the action of ethylene glycol itself.

Finally in experiment 3 the cells are exposed to low sodium chloride content and lack of sodium chloride for short intervals and then cultured in high concentrations of sodium chloride. After both treatments nerve is induced, indicating that the very low concentrations of sodium chloride result in some alteration in the cell surfaces so that induction occurs after the cells are returned to the higher concentration of sodium chloride. It is interesting that the treatment with lack of sodium chloride results in the same type of induction as with sucrose (experiment 3).

DISCUSSION

Clearly substances such as sucrose do not induce by themselves but rather prepare the cells for induction by the salt solution in which they are cultured. The induction by the salt solution is proportional to the concentration of sodium chloride. Do all or most of the so-called inductors act in the same manner as sucrose? Since all the experiments on induction have been carried out in Holtfreter's solution containing a concentration of sodium chloride of 3.4 mg./ml., possibly all the inductions obtained by various compounds and mixtures may be attributed to Holtfreter's solution.

The above possibility is reinforced by the investigations of Barth (1941) and of Holtfreter (1944), which show that Holtfreter's solution will induce neural tissue in *Ambystoma maculatum* and *Ambystoma opacum* presumptive epidermis without benefit of additives of any sort. This is a clear-cut demonstration that Holtfreter's solution is an adequate inductor of nervous tissue. Thus, any compound or mixture claimed as an inductor when used in Holtfreter's solution or its equivalent may merely be preparing the cells for induction by the salt solutions. In order to prove that any substance is an inductor it will be necessary to show that induction occurs during the time of treatment and not after the cells are returned to a salt solution.

In view of the wide variety of compounds and mixtures which have been claimed as inductors, it does seem more reasonable to suppose that all induction is brought about by the ions in the salt solutions used for the culture of presumptive epidermis. If so, we can begin to think more clearly about the mechanism of induction by ions instead of trying to make sense out of the disorderly array of so-called inductors.

As far as normal induction by the mesoderm is concerned, it may now be suggested that the mechanism of normal induction is by ways of ions. For example, we find that cultures of mesoderm mixed with ectoderm prepared from lateral blastoporal lips contain functional nerve and pigment cells as well as muscle and mesenchyme. Thus, mesoderm induces nerve and pigment cells under the conditions of our experiments. However, if the sodium chloride content of our salt solution is reduced to 2.57 mg./ml. no nerve nor pigment cells are induced but muscle and mesenchyme differentiate normally. Thus, the normal induction of nerve and pigment cells by mesoderm is dependent upon a high concentration of sodium chloride. This suggests that the role of the mesoderm during normal gastrulation is to prepare the presumptive neural plate for induction by the ions

present in the blastocoel fluid. This preparation may simply consist in an increase in permeability so that the ions penetrate. Experiments designed to test the above suggestion are in progress.

If we accept the idea that basically induction is brought about by ions, then we have first the problem of which ions in the salt solution are necessary. Previous experiments have shown that Na^+ , K^+ , Ca^{++} , Mg^{++} and HCO_3^- can induce (Barth and Barth, 1963, 1964 and 1965). Secondly, how do the ions act inside the cell to induce cellular differentiation? A previous study of ion induction (Barth, 1965) showed a correlation between the effects of ions as inductors and their effects on the electrophoretic mobility of DNA. Possibly the ions in our salt solution act directly upon DNA complexes.

SUMMARY

1. An analysis of the mode of action of sucrose as an inductor of the presumptive epidermis of the *Rana pipiens* gastrula leads to the conclusion that sodium chloride is the actual inductor.

2. After treatment with sucrose, induction is proportional to the concentration of sodium chloride in the culture medium. After treatment with sucrose, induction is proportional to the length of exposure to a solution containing 3.4 to 5.15 mg. sodium chloride per ml.

3. It is concluded that sodium chloride in concentrations of from 3.4 to 5.15 mg./ml. is an adequate inductor, while in concentrations from 2.00 to 2.57 mg./ml. sodium chloride does not induce but will sustain the differentiation of various cell types after induction.

4. It is suggested that normal induction by the mesoderm during gastrulation may be brought about by the ions present in the blastocoel. The hypothesis that ions act directly upon DNA complexes has been advanced in a previous paper on induction.

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THE pH TOLERANCE OF EMBRYOS AND LARVAE OF MERCE- NARIA MERCENARIA AND CRASSOSTREA VIRGINICA

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The tidal estuarine waters that form the principal habitat of most commercial mollusks are some of the most complex environments in nature. Of the various interacting biological, physical, and chemical factors that affect commercial mollusks in these waters, pH has received less attention than any other major factor. Prytherch (1928) measured the pH at several stations in Milford Harbor and the Milford area of Long Island Sound. He found a pH range during the day from 7.2 to 8.4 and observed that oysters spawned at pH 7.8 to 8.2. Prytherch concluded that low pH inhibited oyster spawning and that oysters in Milford Harbor spawned at high tide because this was the only tidal stage at which the pH was between 7.8 and 8.2. Korringa (1940) quoted Gaarder (1932) and Gaarder and Spärck (1932) who found that larvae of *Ostrea edulis* died when the pH in their oyster polls exceeded 9.0.

In laboratory experiments, Loosanoff and Tommers (1947) found that adult American oysters, *Crassostrea virginica*, kept in pH 4.25 remained open, on an average, 76% of the time, but pumped only 10% as much water as did the controls. Oysters kept at pH 6.75 and 7.00 initially pumped more vigorously than the controls but the rate of pumping later decreased to less than that of the controls.

Although the pH of the open ocean usually ranges from 7.5 to 8.5 (the higher values are at the surface during active photosynthesis), the pH in tidepools, bays, and estuaries may decrease to 7.0 or lower due to dilution and production of H_2S (Sverdrup, Johnson and Fleming, 1942). These inshore areas constitute a major portion of the habitat of commercial bivalves, and Davis and Calabrese (1964) suggested that these regions may be exceedingly important also as the nursery grounds for the larval stages. Since clam and oyster larvae must, at times, encounter a wide range of pH in their natural habitat, it is possible that success or failure of recruitment in some areas may be determined by variations in pH. The present studies were designed to determine the pH tolerance of the embryonic and larval stages of hard clams (*Mercenaria mercenaria*) and American oysters (*Crassostrea virginica*) under laboratory conditions.

METHODS

The methods at this laboratory for maintaining spawners and obtaining fertilized eggs throughout the year have been described previously (Loosanoff and Davis, 1963). The effect of pH on the percentage of eggs of clams or oysters that develop into normal straight-hinge larvae was determined by placing a known number of fertilized eggs (usually 10,000 to 15,000) into each of a series of 1-liter polypropylene beakers of filtered, ultraviolet-treated sea water (salinity $27 \pm 0.5\text{‰}$).

The pH of duplicate cultures was adjusted with HCl or NaOH to each of the following levels: 6.00, 6.25, 6.50, 6.75, 7.00, 7.50, 8.00, 8.25, 8.50, 8.75, 9.00, 9.25, and 9.50. Finally, one pair of cultures retained at the pH of our laboratory sea water (7.40–7.70) served as controls. All cultures were kept in a constant-temperature bath at $25^{\circ} \pm 1^{\circ}$ C. After 48 hours at the experimental conditions, the larvae from each culture were collected on a stainless steel screen. The larvae were resuspended in a 250-ml. graduated cylinder and, after thorough mixing, a

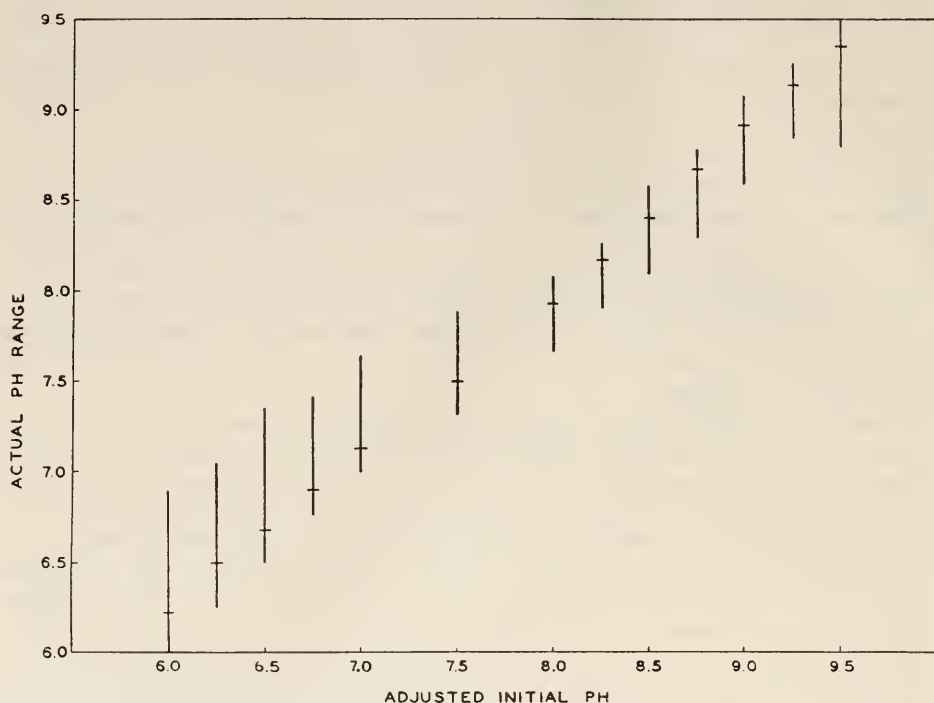


FIGURE 1. Maximum range of pH (vertical line) and average pH (horizontal bar) for each initial pH. The "adjusted initial pH" was established at the beginning of each experiment, and readjusted to this level at 12-hour intervals, by the addition of HCl or NaOH.

4-ml. sample was withdrawn and preserved in 5% neutral formalin. The larvae from each sample were then counted and the number of larvae developing normally at each pH was calculated as a percentage of the number of larvae developing normally in control cultures.

To ascertain the effect of pH on survival and growth, a known number of larvae (usually 8000 to 12,000), which had been reared to the 48-hour straight-hinge stage in our normal sea water (pH 7.40–7.70), was placed into each of the series of cultures. The sea water in these beakers was changed every second day and supplemental food, consisting of a mixture of *Isochrysis galbana*, *Monochrysis lutheri*, and *Chlorella* sp. 580,¹ was added to each beaker daily. The pH of each

¹ *Chlorella* sp. (Indiana U. Collection #580).

culture was adjusted to the desired level immediately after each change of sea water by the addition of an appropriate amount of HCl or NaOH. In experiments with clam larvae it was necessary to add a standard dose (50 ppm.) of Sulmet² at each change of sea water to prevent disease-induced mortality that was not a direct result of the pH being tested. Since buffers were not used, it was necessary to measure and readjust the pH at approximately 12-hour intervals using a line-operated, solid-state pH meter³ having a readability of 0.02 pH unit and a repeatability of 0.01 pH unit. The range and average for each initial pH are shown in Figure 1.

Experiments with clam larvae were terminated after 10 days at the experimental pH levels, when the larvae were 12 days old, because at favorable pH the majority of the larvae had completed larval development and metamorphosis. For similar reasons experiments with oyster larvae were discontinued after 12 days at experimental pH levels, when the larvae were 14 days old.

Quantitative samples were taken from each culture at the termination of an experiment to determine the percentage of larvae surviving and their increase in mean length. In each of these samples, all survivors were counted and 50 clam larvae or 100 oyster larvae from each sample were measured to the nearest 5 μ . The increase in mean length of larvae during the test period was calculated for each pH as a percentage of the increase in mean length of larvae in the control cultures.

The method for determining the number of larvae surviving or the percentage of bivalve eggs developing into normal straight-hinge larvae is accurate to approximately $\pm 10\%$ (Davis, 1958). Differences of less than 20% in the percentage of eggs developing normally or of larvae surviving a treatment are, therefore, considered insignificant.

Five experiments were with clam larvae and four with oyster larvae. In the first three experiments with clam larvae a standard technique was being developed and various buffer systems were being tested. In these initial experiments citric acid, monobasic potassium phosphate, dibasic sodium phosphate, and Tris (hydroxymethyl aminomethane) were used as buffers in an attempt to stabilize the pH at desired levels. The phosphates and citric acid were not effective in maintaining pH levels below 7.00; these buffers also appeared somewhat toxic to clam larvae. When concentrations of these buffers were high, a white flocculent precipitate was formed in the cultures. Tris was of some help in maintaining relatively stable pH levels above 7.00, but was toxic at pH 8.50 or higher, even though apparently nontoxic at levels below 8.50. A precipitate which adhered to the sides and bottom of the beakers was formed at pH 9.50, with or without Tris. The results of these preliminary experiments are not included in our graphs.

Effect of pH on embryonic development of clams and oysters

The number of clam eggs developing normally within the pH range from 7.00 to 8.75 or of oyster eggs within the range from 6.75 to 8.75 did not vary significantly (Fig. 2). The number of both clam and oyster eggs that developed normally at pH 9.00 was greatly reduced and at 9.25 to 9.50 almost none developed.

² Sulmet (Sodium sulfamethazine)—Trade name of American Cyanamid.

³ Instrumentation Laboratory's Model 165 LAB-omatic.

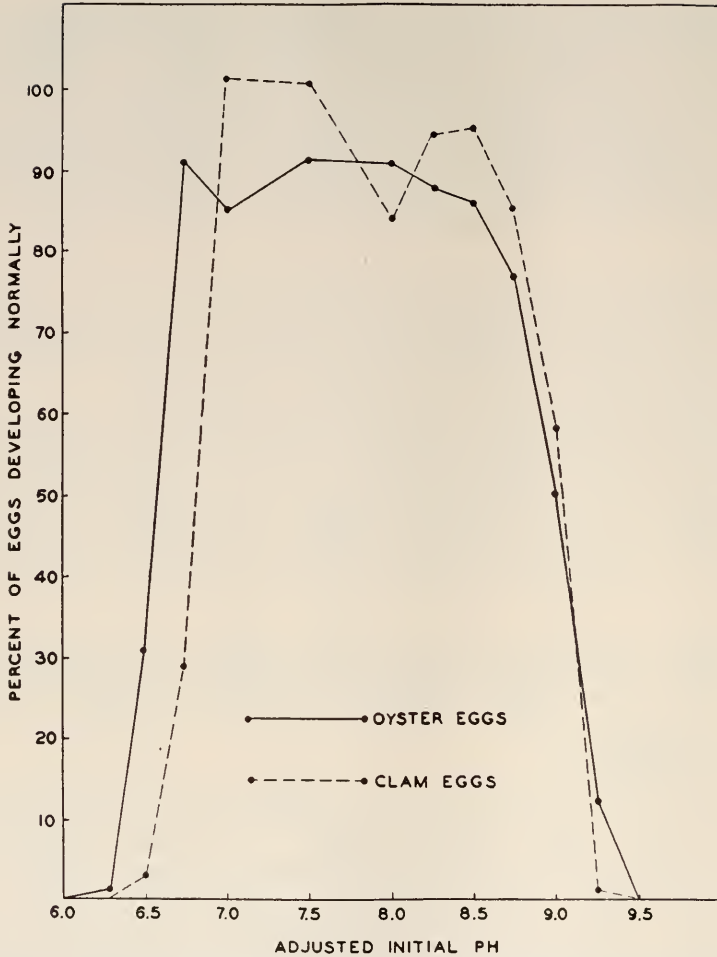


FIGURE 2. Percentage of clam and oyster eggs that developed into normal straight-hinge larvae at different pH levels, expressed as a percentage of the number developing into normal larvae in control cultures.

Clam eggs apparently were not able to tolerate pH values as low as did oyster eggs. At pH 6.75 only 29.5% of the clam eggs developed but 92.4% of the oyster eggs developed normally.

Effect of pH on survival of clam and oyster larvae

Survival of both clam and oyster larvae was approximately normal throughout the pH range from 6.25 to 8.75 (Fig. 3). Oyster larvae were somewhat more tolerant, however, of low pH than clam larvae. At pH 6.00, for example, 21.5% of the oyster larvae survived, but all of the clam larvae died. Survival of both clam and oyster larvae increased sharply from 20% or less at pH 6.00 to approxi-

mately 70% at 6.25 and decreased sharply from 70% or better at pH 8.75 to approximately 40% at 9.00. Most of the larvae lived a few days at pH 9.00 although eventually more than 50% died. No larvae of either species survived at 9.25 and higher.

Effect of pH on growth of clam and oyster larvae

The pH range for normal growth was 6.75 to 8.50 for clam larvae and 6.75 to 8.75 for oyster larvae (Fig. 4). The range for normal growth was, therefore, slightly narrower than the range for normal survival. The rate of growth of clam larvae was most rapid at pH 7.50 to 8.00, whereas oyster larvae grew most rapidly at 8.25 to 8.50. Although oyster eggs and larvae survive at lower pH levels than clam eggs and larvae, the optimum pH for growth of oyster larvae was somewhat

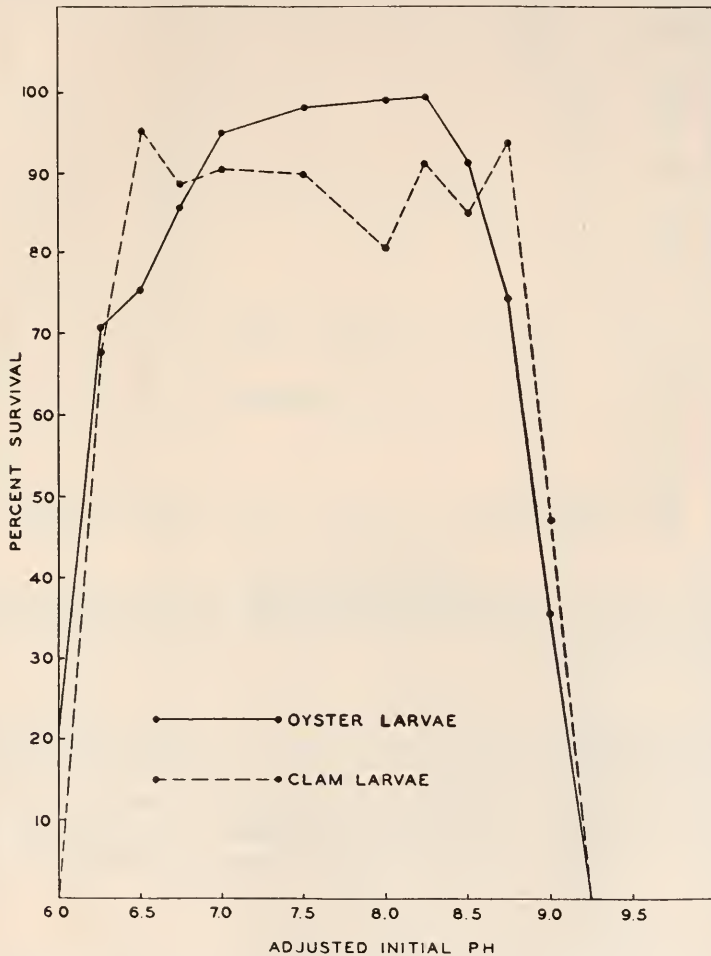


FIGURE 3. Percentage of clam and oyster larvae that survived at different pH levels, expressed as a percentage of survival in control cultures.

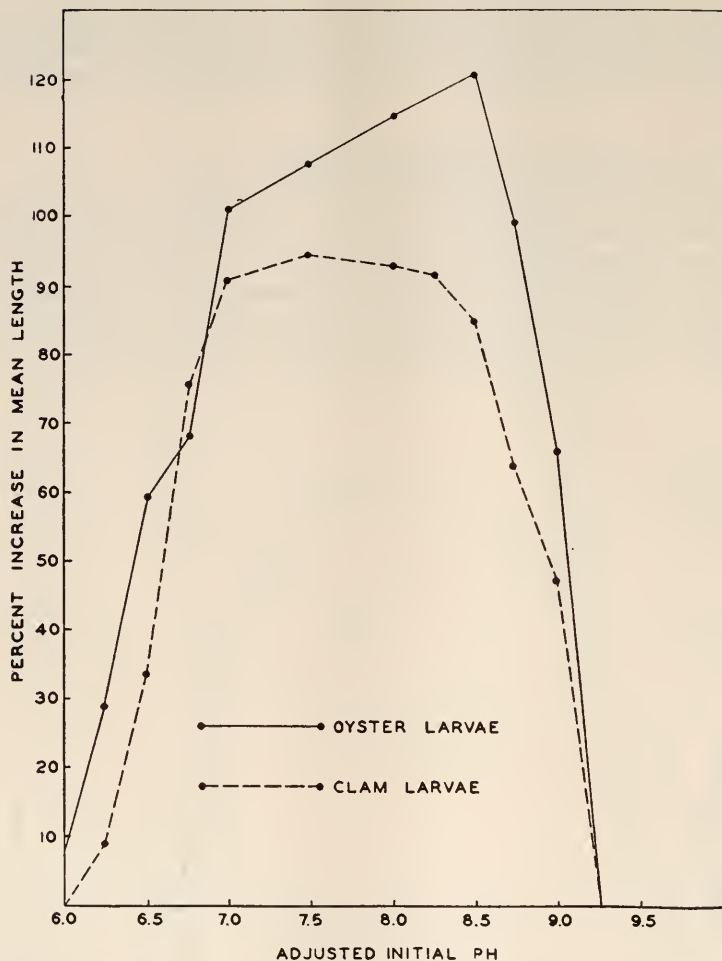


FIGURE 4. Increase in mean length of clam and oyster larvae at different pH levels expressed as a percentage of the increase in mean length of larvae in control cultures. Fifty clam or 100 oyster larvae were measured from each of duplicate cultures at each pH in each of two or more replicate experiments.

higher than the optimum for clam larvae. The rate of growth for both clams and oysters varied only slightly within the pH range 6.75 to 8.50, but below pH 6.75 the rate of growth decreased rapidly. The rate of growth also decreased rapidly at pH values above 8.75 for oysters and above 8.50 for clams. Since the empty shells of dead larvae were not dissolved at the higher pH levels, it was possible to measure them. At pH 9.00 some increase in length had taken place before the larvae died, but at 9.25 to 9.50 there had been no growth.

Implications for distribution and survival in nature

The failure of bivalve larvae to survive and grow at low pH levels did not appear to be an indirect result of the effect of pH on the algal cells added as food. That

the food cells were not destroyed by low pH and that they remained in suspension was shown by the fact that they were ingested by the larvae. Since the larvae were fed supplemental food daily, starvation was unlikely even if some algal cells were destroyed. Even those larvae that survived at low pH and had food in their stomachs, however, did not grow appreciably. Gray (1922) found that movement of gill cilia of mussels was more readily inhibited by weak acids which entered the ciliary cells than by strong acids which do not enter the cells readily and, conversely, that weak bases were more efficient restoratives of ciliary movement than strong bases. Because some food was ingested, even at our lowest pH levels, it seems unlikely that failure of these larvae to grow can be attributed to the effect of pH on ciliary movement.

It should be emphasized that clam larvae can survive at pH levels lower than those at which clam eggs can develop normally (Fig. 5). The range for normal

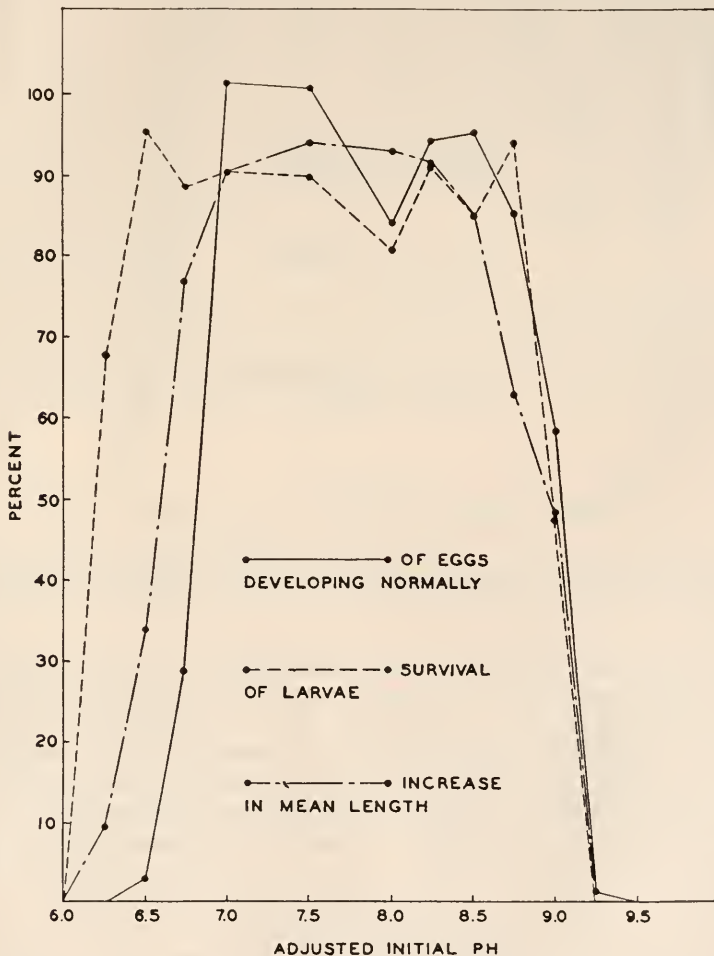


FIGURE 5. The pH tolerance of clam embryos and larvae as indicated by percentage of eggs that developed normally, survival of larvae, and increase in mean length of larvae.

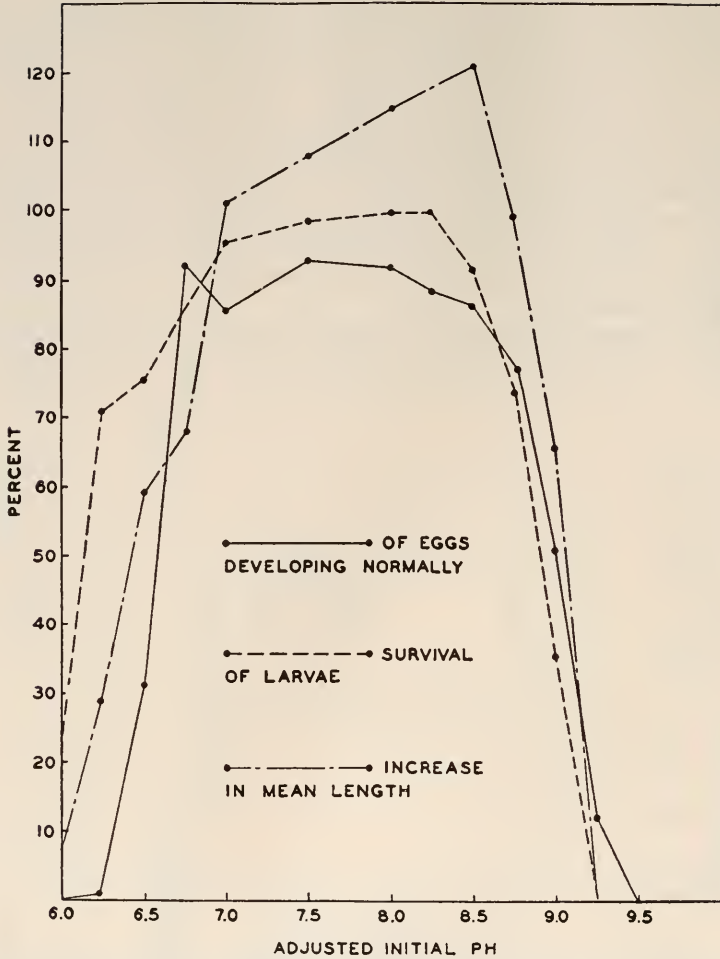


FIG. 6. The pH tolerance of oyster embryos and larvae as indicated by percentage of eggs that developed normally, survival of larvae, and increase in mean length of larvae.

survival of larvae was 6.25 to 8.75, whereas the range for normal embryonic development was only 7.00 to 8.75. In environments with a pH below 7.00, failure of clam eggs to develop normally would be the factor that would limit recruitment of this species. At pH levels 9.00 and above the percentage of clam eggs developing normally, the percentage of larvae surviving, and the percentage increase in mean length all decrease abruptly, so that at high pH levels all three aspects of development limit recruitment of the species. Variations in the percentages of eggs developing normally and of larvae surviving at pH levels between 7.00 and 8.75 were erratic, but all fell within the $\pm 10\%$ confidence limits of our method. Although the pH ranges for normal survival of clam larvae were 6.25 to 8.75 and those for normal rate of growth were 6.75 to 8.50, the optimum for growth was 7.50 to 8.00. The differences in rates of growth at pH 6.75 to 8.50, however, were slight enough

to be negligible in recruitment of this species in nature. The pH of our laboratory sea water (7.40–7.70) was close to optimum for growth of hard clam larvae.

Oyster larvae, like clam larvae, survived at lower pH levels than those at which the eggs developed (Fig. 6). At pH 6.00 none of the oyster eggs developed normally, but 21.5% of the larvae survived. At pH 6.25 the percentage survival of larvae increased sharply, but the increase in the percentage of eggs developing normally was negligible. Most of the oyster eggs developed normally at pH 6.75, whereas a pH of 7.00 was required for most clam eggs to develop. Oysters, therefore, should be able to penetrate into areas of lower pH than clams could tolerate. The range for normal survival of oyster larvae was pH 6.25 to 8.75, and the range for a normal rate of growth of the larvae was 6.75 to 8.75. The optimum pH for growth of oyster larvae, however, was 8.25 to 8.50, *i.e.*, both the optimum and the upper limit for normal growth were somewhat higher than for clam larvae. As with clams, however, the percentage of eggs developing normally, the percentage survival of larvae, and the rate of growth all decrease rapidly at pH 9.00 and above. Since oyster larvae at pH 8.00 to 8.50 outgrew the oyster larvae in the control cultures (pH 7.40–7.70), it was apparent that the pH of our normal laboratory sea water was somewhat too low for the most rapid growth of these larvae.

It can be concluded that for successful recruitment of clams and oysters the pH of the tidal estuarine waters that form their principal habitat must not fall below 7.00 for clams or 6.75 for oysters for any appreciable time. Moreover, neither species could reproduce successfully in waters where the pH remained appreciably above 9.00.

Laboratory experiments have shown that high concentrations of silt can lower the pH of our sea water to 6.40, or below the lower limit for normal development of eggs of hard clams and oysters. It is apparent, therefore, that heavy siltation, or any pollution that can change the pH of tidal estuarine waters, could cause failure of recruitment of hard clams and oysters.

SUMMARY

1. The pH range for normal embryonic development of oysters was 6.75 to 8.75, and for clams, 7.00 to 8.75.

2. More than 68% of the larvae of both clams and oysters survived at pH 6.25 to 8.75. The lower pH limit for survival of oyster larvae was 6.00 and for clam larvae, 6.25.

3. The pH range for normal growth was 6.75 to 8.50 for clam larvae and 6.75 to 8.75 for oyster larvae. The rate of growth of both species dropped rapidly at pH levels below 6.75.

4. The optimum pH for growth was 7.50 to 8.00 for clam larvae and 8.25 to 8.50 for oyster larvae.

5. At pH 9.00 to 9.50 the percentage of eggs that developed normally, the percentage of larvae that survived, and the percentage increase in mean length of both species decreased rapidly.

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⁴ Reviewed by Korringa, 1940, cited above.

DESCRIPTION OF A ZOOCHLORELLA-BEARING FORM OF
EUPLOTES, E. DAIDALEOS N. SP. (CILIOPHORA,
HYPOTRICHIDA)

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Marine and fresh-water species of *Euplotes* are numerous and widely distributed throughout the world. Reviews and historical accounts have been given by Kahl (1932), Pierson (1943), Bovee (1957), Tuffrau (1960), Wichterman (1964) and others. In spite of a great deal of morphological, genetic and cultural work, the taxonomy of this genus is still in an unsettled condition due to variability within species, conjugation between recognized different species, failure of morphologically similar forms to mate and utilization of perhaps unreliable criteria in species differentiation. Moreover, it seems reasonable to believe, as Bovee has suggested, that morphological variation and speciation have followed after reproductive and physiological variation in this genus; hence, the obvious difficulty of dealing with this interesting group of ciliates and the need for the employment of a variety of characters in the recognition of species. A great advance in the analysis of the genus *Euplotes* was made by Tuffrau in his application of the following combination of characters: (1) the number of latero-dorsal kineties, (2) the pattern of the argyrome on the dorsal surface, (3) the number of frontoventral cirri and (4) the shape of the meganucleus in the vegetative state. He based his own extensive revision of the genus on these specific traits. Other students would extend diagnostic criteria to further features, particularly characters relating to the peristome (buccal cavity). Undue reliance on argyrome traits alone can be misleading. For example, the number of kineties in a given species may be variable, as in *E. tuffraui* (Berger, 1965), *E. vannus* (Dusenberry, 1966) and one stock of *E. crassus* (Dusenberry, 1966). In addition, there may be several species possessing the same number of kineties and a somewhat similar dorsal argyrome pattern (Tuffrau, 1960). Recently evolved species may have argyrome patterns very similar to, if not identical with, the ancestral forms, so that it may be very difficult to decide whether or not a given variant is a separate species. The most recently described species, to the writers' knowledge, are *E. leticiensis* Bovee (1957), *E. neapolitanus* Wichterman (1964) and *E. tuffraui* Berger (1965). Borror (1962) has re-described *Euplotes minuta* Yocum. Wichterman reports (1964, pp. 368-369) that "Vacelot (1961) described what he believed to be a new marine species which he named *E. psammophilus*, from *Amphioxus*-sand near Marseilles; but his brief description and poor figure are inadequate to set the species on a firm foundation."

MATERIALS AND METHODS

Collections of samples from a small, permanent, artificial fresh-water pond in the Biological Gardens on the campus of the University of Pennsylvania have con-

sistently yielded over the past seven years specimens of a green species of *Euplotes* which is here named and described as *E. daidaleos* n. sp.¹ Not every collection was positive but there has been no difficulty in securing material from this source. The green *Euplotes* is never present in large numbers in a freshly collected sample and it is often found together with one or more colorless species of *Euplotes* from which it is easily distinguishable because of its different size and its characteristic bright green color. However, when there are few algae in *E. daidaleos* it can be confused with the colorless species which may have ingested green organic food. The green species is not difficult to maintain in the laboratory and thrives on the usual ciliate culture media—hay infusion, Cerophyl infusion, malted milk, powdered milk, rice grains in boiled pond water, etc.

Animals were studied alive, slowed down by 0.05% nickel sulfate or by methylcellulose, as well as in fixed and stained preparations. Many cytological techniques were employed, the most useful being (1) formalin-vapor fixation followed by Bouin's and stained by the Holmes silver technique (Figs. 1–3), (2) the Chatton-Lwoff silver impregnation (Figs. 4, 5 and 8), and (3) Perenyi fixation with carmine-piconigrosin staining (Figs. 6 and 7). All three methods have been described in detail in a previous paper (Diller, 1966).

RESULTS

Size and shape

This fresh-water species averages $92\ \mu$ in body length and $57\ \mu$ in width, for vegetative non-dividing individuals. A range of $77\ \mu$ to $119\ \mu$ in length and $43\ \mu$ to $80\ \mu$ in breadth is encountered. Dividing animals are somewhat larger, as might be expected, averaging $102\ \mu$ in length and $59\ \mu$ in width. Exconjugants are shorter and more rounded, averaging $80\ \mu$ long and $55\ \mu$ wide. *E. daidaleos* is definitely smaller than the common colorless species with which it is commonly found in nature. As is true of all species of *Euplotes*, *E. daidaleos* has a very strongly flattened body and is oval to rounded in face view. The anterior margin, bearing a low collar, is truncated, while the posterior end tends to be bluntly pointed (Figs. 1, 2 and 3). The right anterior margin is straighter than the corresponding corner on the left side which is more rounded. The dorsal surface is slightly convex and bears a constant number of ridges. The ventral surface is flatter and also is equipped with ridges of a characteristic nature. The collar carries the transverse anterior adoral membranelles.

Surface organelles

The peristome is capacious and tapers to a narrow funnel slightly behind the middle of the body, at the cytostome (Figs. 1, 2 and 8). It is bordered on its left and anterior margins by the adoral zone of membranelles (AZM). These are small and close-set in the cytostomal region, becoming wider and larger anteriorly.

¹ The much-studied genus *Euplotes* with its numerous species and many synonyms offered a problem in the selection of an appropriate name for a new species. The term "daidaleos" was finally selected after reference to Brown (1954). He (p. 742) gives the meaning of the Greek word daidaleos as "dappled, spotted." It has an additional meaning (daidalos, p. 104): "cunningly or skilfully made in the manner of Daedalus, the Athenian artificer." The species name seemed fitting, especially so because of the Greek origin of the word *Euplotes*.

There are approximately 40–45 units constituting this structure. Posteriorly, the AZM is opposed by the prominent band of cilia in the mouth area (Figs. 1 and 2) which is known as the undulating or paroral membranelles. The exact conformation of the peristomal cavity is a little difficult to determine but can be analyzed satisfactorily from silver and from picronigrosin preparations. The right edge is

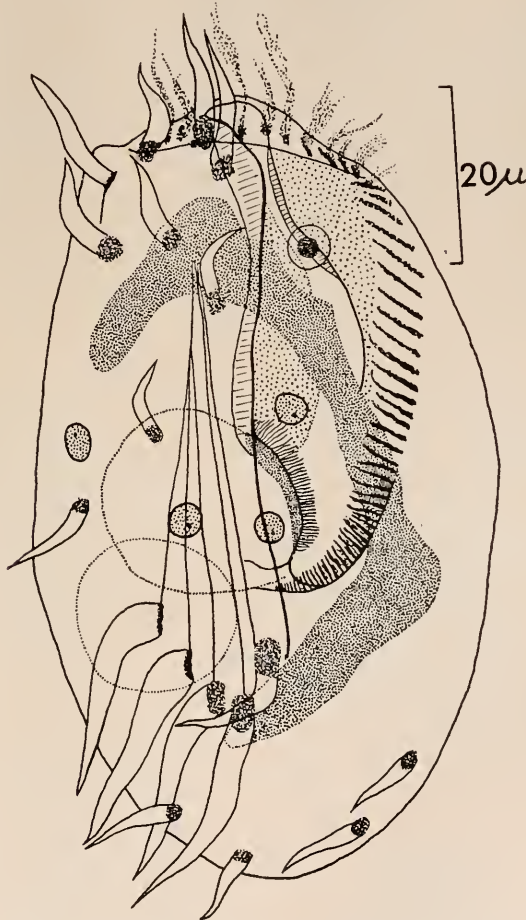
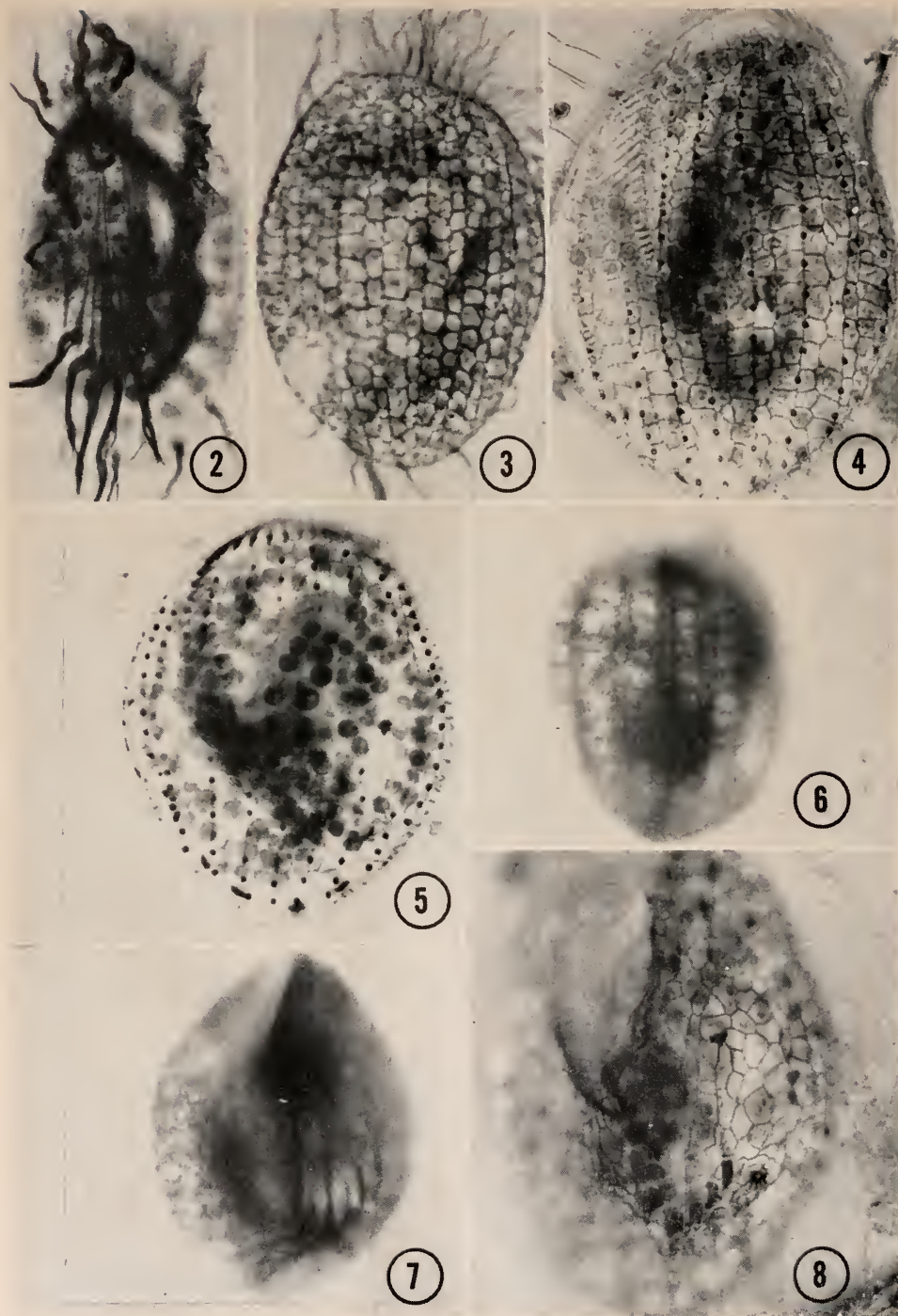


FIGURE 1. Semi-diagrammatic drawing of *Euplotes daidaleos* n. sp. from the ventral side, showing structural features. Taken from a fixed specimen, stained with Holmes silver. Note the collar at the anterior margin of the body bearing some of the adoral membranelles (AZM) which continue along the left wall of the buccal cavity (peristome) to terminate at the cyto-stome; the paroral membranelles near the base of the peristome on its right wall; the peristomal plate extending along the left wall of the peristome; a bulge on the opposite wall of the peristome occupying a considerable portion of the roof of it; an irregular channel between these two thickened areas of the peristomal roof; the endoplasmic sac (corresponding to the level of the paroral membranelles); the contractile vacuole just posterior to the sac; the meganucleus; the micronucleus; the eighteen cirri (six frontals, three smaller ventrals, five anals and four caudals); the "neuromotor" fibers from the bases of the anal cirri and the four zoochlorellae in the cytoplasm.



FIGURES 2-8.

almost straight, being formed by a continuation of the heavy ridge which runs anteriorad from the left side of the medial anal cirrus. This border is undercut by a shallow furrow so as to form a narrow longitudinal lip which is usually a little wider at its anterior and posterior limits than in its center. The lip continues posteriorly over the paroral membranelles to form the posterior border of the peristome, the funnel of the mouth. Along a good part of the right wall of the peristome is a shallow bulge (Fig. 1, stippled) which continues over part of the roof of the peristomal cavity from near the anterior end to about the middle of the paroral membranelles. Opposite this bulge is another thickening of the left wall and roof of the peristome, the so-called peristomal plate (Fig. 1, also stippled). It bears a very small lip. A trough or channel is formed in the roof of the peristome by the space between these two thickenings. The channel is expanded anteriorly at the level of the collar and also posteriorly in the region where actual ingestion occurs. In other species of *Euplotes* we have seen small food organisms entrapped in the anterior expansion which seems to serve as a food-collecting mechanism. However, we have not noticed any collection of food material in this structure in *E. daidaleos*.

The 18 cirri are very constant in number and position. In comparison with colorless species, one has the impression that those of *E. daidaleos* are longer and slenderer. Very rarely five caudal cirri (instead of four) are present. These may represent reorganizations from division of the cell. Also, in exconjugants (at certain stages) only five instead of six frontal cirri are present. The usual "biological variation" can account for the infrequent cirral anomalies which affect the caudals, mainly. The number and position of the cirri are as follows: six frontals in three rows of two each, three ventrals in an oblique row on the right ventral

EXPLANATION OF FIGURES 2-8

Euplotes daidaleos. Photomicrographs. Specimens shown in Figures 2 and 3 were fixed in formalin vapor and Bouin's, followed by the Holmes silver technique. Figures 4, 5 and 8 are specimens fixed in Champy, DaFano and treated with the Chatton-Lwoff silver impregnation. Figures 6 and 7 are exconjugants fixed in Perenyi's fluid and stained by Grenacher's alcoholic borax carmine and picronigrosin.

FIGURE 2. Ventral view. Typical individual showing the locations of all of the eighteen cirri, the shape of the meganucleus, the fibrils from the anal cirri, the AZM, the paroral membranelles and some of the zoochlorellae (approx. 50). $\times 700$.

FIGURE 3. Ventral view, with the dorsal argyrome in focus. The alternating rows of narrower and wider polygons or "compartments" are easily seen. The dorsal bristles are not prominent. $\times 615$.

FIGURE 4. Dorsal view. Most of the rows of dorsal bristles are visible—located in meridians on the right side of the wider "compartment" rows. $\times 650$.

FIGURE 5. Dorsal view. All nine rows of latero-dorsal bristles are visible. The zoochlorellae are prominent, as are the elongated basal plates of the four caudal cirri. $\times 645$.

FIGURE 6. Dorsal view. The rows of narrow polygons contain or overlie protoplasmic constituents (probably mitochondria) which are thus organized differently from their condition in the wider "compartment" rows so as to give a banded organization to the dorsal cortex. $\times 600$.

FIGURE 7. Dorsal view, with the ventral surface in focus. The stained granules noted in Figure 6 are differentially distributed in the ventral cortex also, outlining the ventral ridges. $\times 625$.

FIGURE 8. Dorsal view, with focus on the ventral surface to show the nature of the ventral argyrome pattern. To the right of the bases of the anal cirri is the contractile vacuole pore. $\times 690$.

surface, five large anals (four in line on the right side and the medial one slightly anterior to the last of this line) and four small caudals. The two right caudals regularly tend to be finbriated.

Ridges are present on both dorsal and ventral surfaces. On the latter there are six ridges supporting the grooves in which the anal cirri lie. The ridges are of varying lengths and prominence (Fig. 7). Starting from the right side, nos. 1, 4 and 6 are the longest. The latter participates in the formation of the major ridge which becomes the right wall of the buccal cavity. The ridges on the dorsal side are definite, parallel, equidistant and each bears a row of dorsal bristles. More will be said about these "latero-dorsal" bristle rows in connection with the discussion of the argyrome.

The argyrome

Various silver techniques (Figs. 2, 3, 4, 5 and 8) and, under certain conditions, the picronigrosin stain (Fig. 6) demonstrate the "latero-dorsal kineties," the dorsal bristles and associated structures which may be interpreted as either polygons, fibrils or a meshwork. Exactly what is brought out is somewhat uncertain: kinetosomes, pellicular structures or actual cortical protoplasmic elements. Probably a variety of structures is indeed impregnated and caution is required in interpretation. However, with the use of the standard Chatton-Lwoff technique, *e.g.*, in Figures 4, 5 and 8, striking, reproducible and exceedingly useful preparations can be produced so as to compare and separate different forms of *Euplotes*. Tuffrau has made extensive use of this silver technique in his admirable study of the genus. *E. daidaleos* possesses nine latero-dorsal rows of bristles (Figs. 4 and 5 particularly). Eight of these kineties are on the dorsal surface, the ninth definitely on the ventral surface. In specimens well flattened in gelatine all nine kineties may be visible at practically the same focus. The number of kineties is remarkably constant in *E. daidaleos*: in hundreds of specimens carefully studied only one showed a discrepancy from this number, nine. The number of kinetosomes per row is also fairly constant. Starting with the ventral row, then going to the dorsal side and proceeding to the right edge we have counted the following numbers of bristles per row: 13-16, 7-17, 15-18, 15-19, 17-20, 16-18, 16-18, 13-19, 10-19. The total number of bristles per animal varied from 123 to 158 in the specimens counted. Between the kineties—kinetosomes seem to lie on a "fibril"—are stainable lines (Figs. 4 and 5) which look like cross-connecting fibrils. With the picronigrosin technique (Fig. 3) one gets the impression that this configuration may be shallow polygons of alternating wider and narrower rows (Figs. 3 and 6) of pellicular or of superficial cortical material. The dorsal bristles always lie to the right of a wide row and to the left of a narrow row of these polygons. It would appear that the wider and narrower rows may represent some real cytoplasmic difference, since, with the picronigrosin stain (Fig. 6), there is a differential staining reaction, the narrow rows taking up the blue stain much more intensely than the wide rows do. Probably, the mitochondria are being stained and this banded effect represents a differential distribution of the mitochondria. One can think of no logical explanation for this condition. On the ventral surface the mitochondria in the ridges take up the picronigrosin intensely (Fig. 7). The argyrome pattern of the ventral

surface (Fig. 8) is more irregular and finer (including the roof of the peristome) than it is on the dorsal surface. As is characteristic of all species of *Euplotes*, the meshwork immediately surrounding the contractile vacuole pore, to the right of the anal cirri (Fig. 8), consists of much smaller polygons.

Internal structures

The shape of the C-shaped meganucleus is somewhat variable and not particularly distinctive for *E. daidaleos*. Typically, the anterior arm bends backward but the posterior arm does not curve forward to meet it, so that a symmetrical C is not formed (Figs. 1 and 2). Usually the anterior arm extends forward into a small hump as it becomes drawn out in the main body of the organelle. The position of the micronucleus is very constant, at the anterior curvature of the meganucleus slightly dorsal to it and between it and the AZM. It is a small vesicular structure less than $5\ \mu$ in diameter enclosing a homogeneous endosome.

The presence of symbiotic algae in the cytoplasm of *E. daidaleos* is very characteristic and perhaps a constant feature of this species. The zoochlorellae vary in number from a very few (Fig. 1 contains four) to perhaps 100 (Figs. 4, 5 and 8). Their size ranges from 3 to $5\ \mu$ in diameter. When a culture is dividing rapidly the number of algae is less per host than when the population is static. We do not know whether this species can normally exist without their customary algae; if so, whether they can reacquire their symbionts from the medium. In clonal cultures, all of the individuals possess at least some zoochlorellae under normal conditions. When colorless *Euplotes* and *E. daidaleos* are mixed together, both colorless and green individuals can be recognized as such for many weeks. Just as *Paramecium bursaria* can be freed of its algae, so it might be expected that *E. daidaleos* can be sterilized of its zoochlorellae. However, critical studies have not yet been made on these matters of obligatory symbiosis, mode of "infection" and survival in the absence of algae. It should be noted that exconjugants frequently contain more algae than their non-conjugated associates. This can be explained on the basis of a long reorganization interval between conjugation and the first postconjugant fission during which the algae divide and are not diluted by division of their host cell.

Many of our Holmes silver preparations bring out the five long internal fibers originating at the bases of the anal cirri and converging at the anterior end in the region of the medial frontal cirri (Figs. 1 and 2), as well as other fibers associated with other cirri. We have not been able to detect any structure which could be interpreted as a neuromotorium.

The food material appears to be bacteria, algae, flagellates and small ciliates of the pleuronematid type. Some cultures show bacteria-like bodies in the cytoplasm which resemble Kappa particles in *Paramecium*. They are quite distinct from the mitochondria and are often sharply demonstrated by the Holmes technique. A prominent endoplasmic sac (Fig. 1) is present on the right side of the body. Its left side appears to be very intimately associated with the bases of the paroral membranelles.

Just posterior to the endoplasmic sac is the contractile vacuole (Fig. 1). Its external pore is a fixed spot on the ventral surface.

Life cycle

Conjugation has been observed frequently in non-isolated mass cultures of *E. daidaleos* and in cultures started from a few (8–10) individuals from wild cultures. Studies of the correlation of ciliary and nuclear development in the life cycle of this species have been published elsewhere (Kounaris, 1964; Diller, 1966). These report binary fission and conjugation. Encystment in this species has never been seen by us.

DISCUSSION

Colored species of *Euplotes* have been described as far back as Ehrenberg (1840) who named a form from Berlin *E. viridis*. Stein (1859) presented three figures of forms he called *Euplotes patella*, noting that very frequently their cytoplasm is more or less thickly filled with bright green chlorophyll bodies. He referred to one of these as “disc-forms.” Apparently he felt that there were several types of chlorophyll-bearing *Euplotes*. Stein identified Ehrenberg’s *Euplotes viridis* from Berlin as most probably nothing more than the chlorophyll-bearing form of *E. patella*. Pierson, in her review of species of *Euplotes* closely related to *Euplotes patella*, makes no reference to green *Euplotes*. However, Kahl (1932) listed three colored types: all “formae” of *E. patella*. These are forma *typicus*, 80–100 μ , mostly with zoochlorellae; forma *latus* 90–120 μ , often with zoochlorellae; forma *alatus*, broad form with zoochlorellae. Obviously, he recognized different forms as capable of harboring zoochlorellae. Tuffrau, in his revision of the genus *Euplotes*, does not mention the occurrence of zoochlorella-bearing forms and dismisses Kahl’s alga-bearing formae, together with Ehrenberg’s *E. viridis*, as synonyms of *E. patella*. This decision was made largely on the basis of culture work by Pierson and himself which indicated intra-clonal variation of *E. patella*.

Tuffrau lays particular emphasis on the distinctiveness of the dorsal argyrome pattern of *E. patella*: the alternating wide and narrow polygonal rows. *E. daidaleos* has a dorsal argyrome pattern similar to *E. patella*—perhaps not quite as regular as Tuffrau has shown. His size of *E. patella*—105–145 μ —is greater than we have found for *E. daidaleos*. However, Pierson’s size of *E. patella*—90 by 52 μ —is practically the same as for *E. daidaleos*. Kahl’s *E. patella* is larger.

Most of the published figures and descriptions of *Euplotes* allow for some uncertainty with regard to the detailed structure of the buccal cavity: its wall, relative length and curvature, lips, bulges, channels, plates and membranelles. It is uncertain how similar, or dissimilar, *E. daidaleos* is to other species with respect to the features of the buccal cavity but it does not seem to fit either Pierson’s or Tuffrau’s description of *E. patella* exactly. Pierson has emphasized the desirability of including these features in the diagnostic determination of species of this genus.

Clearly, *E. daidaleos* is closely related to *E. patella* but on the basis of difference in body shape, constant possession of zoochlorellae, smaller size and details of the structure of the peristome, *E. daidaleos* is considered to be a distinct species, hitherto undescribed.

SUMMARY

1. *Euplotes daidaleos* n. sp. is described as a hitherto unrecognized species. It is a fresh-water form found in Philadelphia, Pa., and appears to be closely related

to *E. patella*, differing from it in shape and size, in the possession of zoochlorellae and possibly in the structure of the peristome.

2. Diagnostic characteristics: Fresh-water. Contains zoochlorellae (few to 100). Average length 92 μ , width 57 μ . Body flattened, oval in face view; right anterior margin straighter than the more convex shoulder on the left side; posterior end bluntly pointed. Buccal cavity (peristome) extending slightly beyond middle of body. Adoral membranelles approximately 40, arranged in a smooth curve terminating on the right edge of the collar. Right edge of the peristome an almost straight wall originating as an extension of the ventral ridge at the left of the anal cirri; undercut so as to form a narrow lip, anterior to the paroral membranelles; attached to it and the dorsal wall of the buccal cavity is a low bulge partly occluding the cavity. Between this bulge and the elongated triangular peristomal plate on the left anterior side of the buccal cavity is a trough or channel expanded anteriorly as well as posteriorly in the region of the paroral membranelles. The latter delimit the endoplasmic sac on the right. Eighteen cirri: six frontals, three ventrals, five anals and four caudals, two of the latter on the right side tending to be fimbriated. Meganucleus C-shaped. Nine latero-dorsal rows of bristles, corresponding in position to the dorsal ridges (the left one distinctly ventral). Between each dorsal "kinety" are two rows of alternating wider and narrower polygons, revealed by silver impregnation techniques. The dorsal bristles lie on the right border of the wide rows and to the left of the narrow rows.

3. Conjugation is common in the stocks of *E. daidaleos* examined. Encystment has not been found.

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THE INFLUENCE OF LIGHT ON THE SIZE OF AGGREGATIONS IN *Dictyostelium discoideum*

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Fruiting structures of the Dictyosteliaceae formed in light are smaller and more numerous than those produced in comparable cultures incubated in darkness (Potts, 1902; Harper, 1932; Raper, 1940; Heller and Miles, 1961; Shaffer, 1961; Kahn, 1964). Since the size of a fructification, or sorocarp, is dependent to a large extent upon the number of cells that enter an aggregation, it is logical to seek the bases for this behavior in the aggregative process *per se*. Bonner and co-workers (Bonner and Dodd, 1962; Bonner and Hoffman, 1963) have reported that for certain species of *Dictyostelium* the size of the aggregation territory remains the same in cultures grown under constant environmental conditions. They believe it possible, as does Shaffer for *Polysphondylium violaceum* (Shaffer, 1961), that an inhibitory substance diffuses outward from the first-formed centers and prevents the formation of additional ones, thus determining the disposition of developing aggregations. To this putative factor they have applied the term "spacing substance." More recently, Kahn (1964) has suggested that cell aggregation in *Polysphondylium pallidum* may be inhibited in darkness by a center-suppressing factor, the effect of which is erased by illumination. For a summary of published information and opinion prior to 1962 regarding the process of cell aggregation in the Dictyosteliaceae, the reader is referred to Shaffer's comprehensive review (1962), "The Acrasina."

The effect of different light conditions on the time of aggregation in *Dictyostelium discoideum* has been investigated (Konijn and Raper, 1965). But no studies have been made to determine at what time during the preaggregative stage the existing light conditions influence the number of aggregations formed (and by inference the size of aggregation territories) or the number of fruiting bodies that subsequently develop. The present study attempts to assess the effect of light during the preaggregative stage on the number of aggregations that subsequently arise, and to correlate differences in the size of such aggregations in light and in darkness with possible changes in the activity of, or the cellular responses to, the chemotactic substance(s) secreted by the converging myxamoebae.

MATERIALS AND METHODS

Dictyostelium discoideum Raper, NC-4(H), a haploid strain derived from the diploid stock, NC-4, was the culture most used during this research. The myxamoebae were grown in either light or darkness and on either a solid medium

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(Bonner, 1947) in association with *Escherichia coli* #281, *E. coli* B/r or *Aerobacter aerogenes* #900, or in shaken tube cultures with pregrown *E. coli* B/r according to the technique of Gerisch (1959). The details of harvesting the myxamoebae were given in our previous report (Konijn and Raper, 1965).

Populations of two sizes and densities were employed in the present study:

(1) For investigating the number and size of aggregations that would develop under different light conditions, washed myxamoebae were resuspended in distilled water, or in Bonner's salt solution (Bonner, 1947), at a dilution of 4×10^5 cells/ml. and deposited on non-nutrient agar (1.5%) as 0.1-ml. aliquants. Three such drops were implanted per Petri dish and, after having spread on the agar surface, covered areas 1.5 to 1.9 cm. in diameter. The resultant cell density was usually about 200 to 250 myxamoebae per/mm.², there being some increase in the cell populations after deposition (see Konijn and Raper, 1961).

(2) For investigating the influence of light on cell attraction, *minute* droplets of a much denser suspension (1.5×10^7 cells/ml.) were deposited on a soft gel made with highly purified agar and Bonner's salt solution (Konijn and Raper, 1961). The number of cells per population was 800 to 1600, and the areas covered by the droplets were approximately 0.5 mm. in diameter. Depending upon the time of deposition, populations were designated as either "attracting" or "responding." From 150 to 200 droplets containing attracting populations were first deposited on the surface of an agar plate with hand-drawn micropipettes, while an equal number of similar droplets containing responding populations were deposited later at distances of 400 to 1200 μ from the former. The concentration of the agar used to form the gel was *ca.* 0.5%, or sufficient to yield a rigidity of 35 to 40 grams expressed as the weight required to cause the end of a microscope slide to break the agar surface (Konijn and Raper, 1961). For a particular experiment, a single cell suspension was used as the source of both attracting and responding populations, the suspension being held in a refrigerator until the latter were placed on agar.

The light source employed was "cool white" fluorescent tubes, and the light intensity was *ca.* 60 foot candles at the level of the agar surface on which the myxamoebae were deposited. The incubation temperature was $23 \pm 1^\circ \text{C}$.

RESULTS

1. The influence of light on the number and size of developing aggregations and sorocarps

Myxamoebae of *Dictyostelium discoideum*, NC-4(H), were grown on agar in darkness and harvested in the preaggregative stage. After removal of excess bacteria by centrifugation, the cells were resuspended, deposited on non-nutrient agar and incubated under different light conditions. The total number of fruiting organizations formed within the populations was recorded after 25 hours. As shown in Figure 1, more and smaller aggregations and fruiting structures were formed in constant light than in constant darkness. Since only a small minority of the myxamoebae stayed outside the developing aggregations in either case, such cells could not account for the lower number produced in the dark. Of special interest was the influence of an initial period of dark incubation of varying duration. The number of sorocarps formed in light gradually decreased as an initial dark period

was increased, and the lowest number developed if a dark period optimal for early aggregation (*e.g.*, 8 to 9 hours darkness) preceded exposure to light. Myxamoebae that were transferred from darkness to light shortly before aggregation began, or in the early aggregative stage, usually produced a near maximum to maximum number of fruiting structures, as for example in plates incubated in darkness for 12 or 13 hours. The number of fruiting structures formed under similar light conditions varied considerably in different experiments. After an initial dark period of 14 hours, aggregations in some experiments were already well advanced, and the

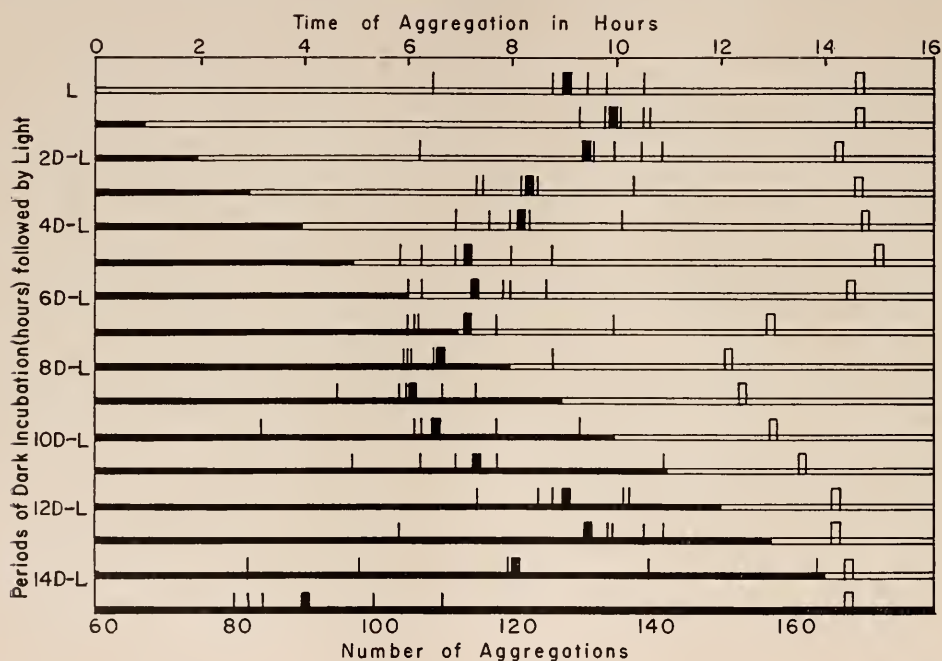


FIGURE 1. The effect of an increasing initial dark period on the number of aggregations and sorocarps in *Dictyostelium discoideum*, NC-4(H). The myxamoebae were grown in the dark on a solid medium. The results of five experiments are included. — Light. ■ Darkness. | Number of aggregations per population in individual experiments. Each mark represents the average of 6 drops. ■ Average number of aggregations per population in all experiments. □ Average time of aggregation.

final count of fructifications in such plates was close to the total number formed in complete darkness. In other experiments, the myxamoebae were in a less advanced stage and in these the number of aggregations was increased substantially by exposure to light. Variation in the diameters of the drop areas due to minor differences in the non-nutrient agar may have contributed somewhat to the inconstant behavior. However, such variation should not have been greater in plates incubated for 14 hours in darkness than in the other series.

The same general approach was used in a second set of experiments except that the myxamoebae were grown in shaken cultures in the dark. In these tests, variation caused by differences in the areas covered by the drops, or by a possible "edge

effect" at the boundaries of the drops, was minimized by counting only the number of fruiting structures in an area 1.0 cm. square at the center of each population. Counts obtained in this way were roughly proportional to those obtained in the earlier experiments when all the sorocarps that developed within a drop were counted (Fig. 2). The onset of aggregation occurred somewhat earlier than with

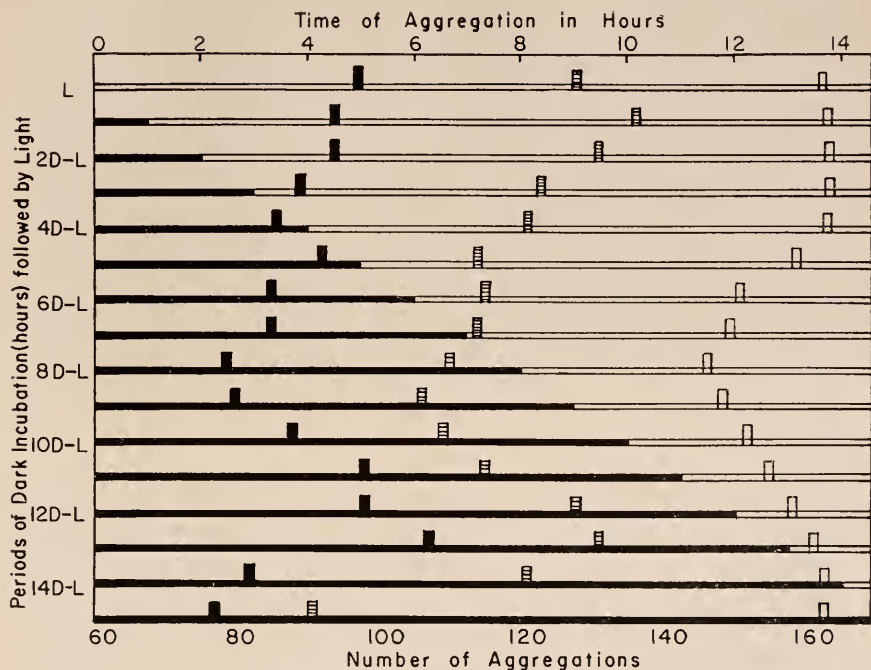


FIGURE 2. A comparison of the numbers of aggregations in entire populations of *Dictyostelium discoideum*, NC-4(H), and from areas 1 cm. square at centers of populations of comparable size and density. The myxamoebae for the latter tests were grown in the dark in a liquid medium. The results of four experiments are averaged. — Light. ■ Darkness. ■ Average number of aggregations in centrally placed areas 1 cm. square. Each mark represents the average of 24 determinations. ▨ Average time of aggregation in the four experiments. ▨ Average numbers of aggregations in entire populations of comparable size and density. (Data from Fig. 1.)

myxamoebae dark-grown on the solid medium, this difference being as much as two hours in cultures incubated in the dark for 5 or 6 hours prior to being transferred to light.

Other species and strains were examined in a third set of experiments. In *Dictyostelium purpureum*, WS 321, and in *Polysphondylium pallidum*, WS 320, the number of aggregations was much reduced when the populations of myxamoebae were transferred from light to darkness at an early aggregative stage. If the cells were first kept in the dark for a few hours and later exposed to light, the number of aggregations increased if the transfer took place at or near the beginning of aggregation. Also, in *D. discoideum*, strain Acr 12, aggregations

occurred earlier and in greater numbers in light than in darkness. Thus inter- and intraspecific differences in response to light must occur since cell populations of low density (200–250 myxamoebae/mm.²) were employed, as for *D. discoideum*, NC-4(H).

2. *The influence of light during the preaggregative stage on the rate of increase in the number of aggregations*

Myxamoebae of *Dictyostelium discoideum*, NC-4(H), were grown in shaken cultures in the dark, harvested, washed and deposited as 0.1-ml. drops on non-nutrient agar. Surveys at half-hour or hourly intervals after the cells started to aggregate indicated that the number of aggregations increased slowly if an initial

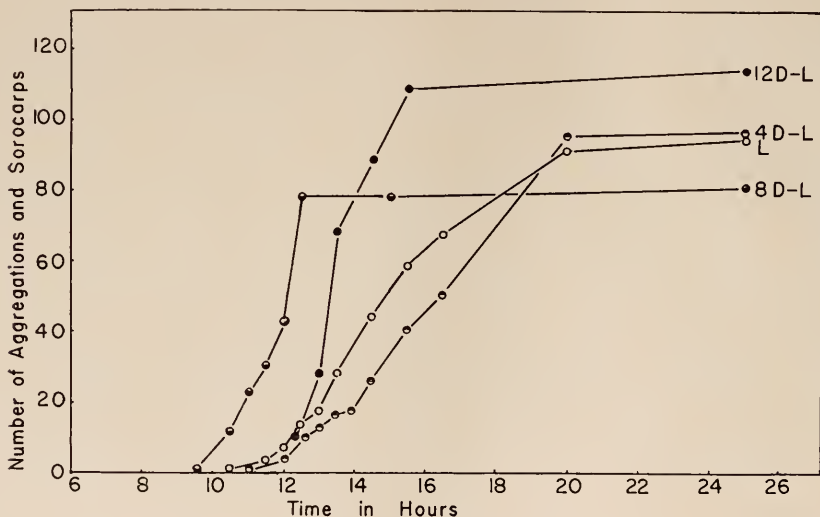


FIGURE 3. The effect of an increasing initial dark period on the rate of increase in number of aggregations. L: Light; D: Darkness; D-L: the drops were in darkness for the number of hours indicated before exposure to the light.

dark period was lacking, or if this was insufficient to induce early aggregation (Fig. 3). After a dark period of 8 hours or longer, however, the number of aggregations increased rapidly, and within a period of one to three hours the final number was reached. Primary aggregations in populations exposed to light after a dark incubation of 10–13 hours were relatively large and the increase in number of sorocarps formed in such plates resulted, in substantial part, from a breaking up of the streams of pre-existing aggregations into smaller pseudoplasmodia. The sample curves presented in Figure 3 are taken from a much more extensive series twice confirmed in which periods of light and dark incubation were varied at increments of less than four hours.

3. *The influence of light on chemotaxis*

Information relating to the phenomenon of chemotaxis was obtained by observing the behavior of small populations of myxamoebae when deposited on the surface

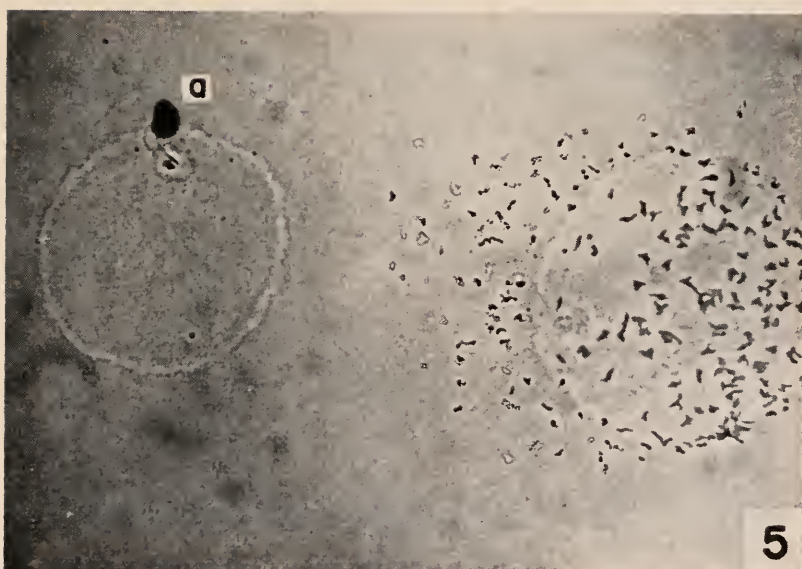


FIGURE 4. A small population of myxamoebae deposited on washed non-nutrient agar of low rigidity. Without attracting forces from the outside the myxamoebae stay inside the boundary of the drop. $\times 140$.

FIGURE 5. Myxamoebae in a responding population attracted outside the boundary of the original drop by an aggregation (a) in the neighboring drop. The myxamoebae move toward the attracting aggregation by wriggling through the agar. The rigidity of the agar surface was the same as in Figure 4. $\times 100$.

of washed, non-nutrient agar of low rigidity (Konijn and Raper, 1961). The soft agar gel provided a means of measuring interpopulational responses since it was rigid enough to keep all the cells within the confines of the drop in the absence of an extraneous stimulus (Fig. 4), but was, at the same time, sufficiently soft to allow the myxamoebae to move outside the drop boundary if attracted by a chemotactic stimulus secreted by a neighboring population. Fortunately for our purposes, the myxamoebae that were attracted beyond the edge of such a drop moved *into* the agar (Fig. 5) and did not return to their "home" drop until its residual cells formed their own aggregates. Thus data relating to the effect of chemotactic stimuli could be obtained by employing droplets of cells of one preaggregative "age" to act as attractors and cells that were less mature in point of time to serve as responders. Drops containing attracting and responding myxamoebae were

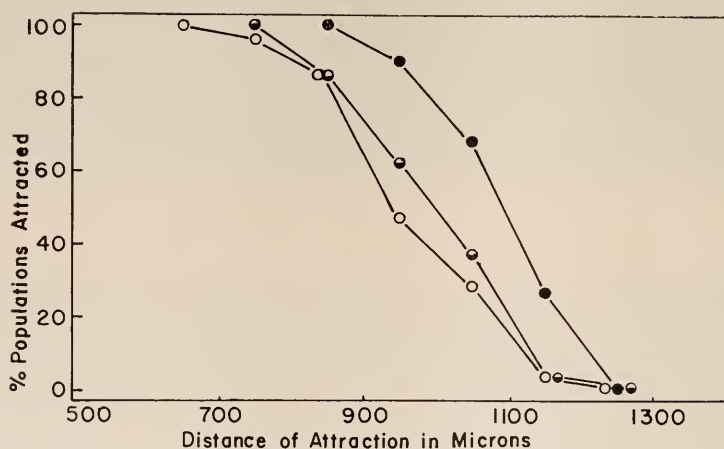


FIGURE 6. The percentages of myxamoeba populations that showed a response to developing aggregations under different light conditions, and the distances between the nearest margins of these populations and the aggregations that attracted them. Graphic representation of data contained in Table I. ○: Constant light. ◐: Constant light, but with plates sealed after the responding drops were deposited on the agar. ●: Constant darkness.

deposited on the agar surface at different distances from each other and the plates were then incubated in either light or darkness. The distance over which attraction could take place was taken as a measure of the strength of the stimulus produced by the population of aggregating myxamoebae. This distance was measured not between the proximal edges of the two drops but from the *center* of the developing aggregation in the attracting drop to the nearest margin of the responding drop. The response was considered as positive when two or more cells moved outside the edge of the responding drop toward the attracting population. Responding myxamoebae rarely moved more than 500 μ outside their "home" drops.

For the actual tests, minute droplets of a dense suspension of cells pregrown on a solid medium were deposited on low-rigidity washed agar and occupied areas approximately 0.5 mm. in diameter. In preliminary experiments, responding populations plated three hours after the attracting populations showed less response

when incubated in the light than in darkness. Fifty per cent of the attracting small populations incubated in the light aggregated after about 8–10 hours, which was on average one hour earlier than those in the dark, and it was thought that responding cells that were themselves nearer in time to aggregation might have an increased sensitivity to chemotactic substances. In order that the “physiological age” of the responding drops in light and in dark might be more nearly identical at the time aggregation began in their counterpart attracting drops, the responding cells to be incubated in the dark were deposited one hour later than those to be incubated in the light. The influence of light on attraction by aggregating myxamoebae is graphically presented in Figure 6. Attraction in the dark was observed to occur, on the average, over a longer distance than in the light (Table I). For example, 50% of the illuminated populations showed a response over a distance of 940 μ , while an equal percentage of those incubated in the dark responded over a distance of 1090 μ .

TABLE I

Attraction of myxamoebae in populations of responding cells by developing aggregations in plates incubated in light, in darkness, and in light with plates sealed. The 2056 populations represented in this table were tested in three different experiments

Distance between aggregations and the responding populations	Light			Light, sealed			Darkness		
	+	–	%	+	–	%	+	–	%
600–700 μ	17	0	100						
700–800 μ	47	2	96	30	0	100	17	0	100
800–900 μ	126	22	85	137	23	86	153	0	100
900–1000 μ	98	111	47	125	77	62	233	25	90
1000–1100 μ	45	118	28	49	84	37	159	76	68
1100–1200 μ	2	61	3	2	57	3	20	58	26
1200–1300 μ	0	27	0	0	22	0	0	33	0

Code: + Test populations responding to an aggregation in the “attracting drop”; – Test populations not responding to an aggregation in the “attracting drop”; % Percentage of test populations responding.

A possible decrease of humidity in plates incubated in the light could have affected the agar surface and consequently limited the movement of the responding cells outside the edge of the drop. For this reason, control plates in the light were sealed with masking tape to prevent evaporation and to reduce any possible gaseous exchange which might influence aggregation, as has been reported by Bonner and Hoffman (1963). The distance over which attraction occurred in sealed plates in three different experiments was equal to or slightly greater than that observed in the unsealed, light-incubated plates, but it was never equivalent to that recorded in the dark (Fig. 6).

DISCUSSION

When an initial dark period optimal for early aggregation in *Dictyostelium discoideum*, NC-4(H), was employed (see also Konijn and Raper, 1965), the number of sorocarps was less than in constant light and greater than in constant

darkness. A further increase in the period of dark incubation resulted in a delay of aggregation and an increase in the number of sorocarps, particularly, if the transfer to light occurred near or at the onset of aggregation. The number of sorocarps, however, was not necessarily related to the time of aggregation. Myxamoebae of *D. discoideum* incubated in continuous light aggregated slightly later than cells kept in darkness, but formed sorocarps more abundantly. Light could exert its effect by a stimulation of center formation, or by an inhibition of the spacing substance (Bonner and Hoffman, 1963), *e.g.*, by reducing the sensitivity of cells to it. It is questionable whether a gaseous spacing substance determines the number of aggregations and consequently influences the number of fruiting structures in this species, for Bonner and Hoffman (1963) noticed that the gaseous spacing substance that has such a pronounced effect on *D. mucoroides* does not affect the myxamoebae of *D. discoideum*, although the latter are able to produce a gaseous substance that influences the spacing in other species.

The increase in the number of sorocarps produced when myxamoebae are exposed to light at an early aggregative stage is dependent, at least in part, on a delicate balance within the aggregations. For example, if populations in the process of aggregating were placed under fluorescent light, the aggregates already formed would sometimes break up into several smaller pseudoplasmodia. At other times the binding forces within the aggregations were strong enough to prevent severance of streams when exposed to light, and no significant increase in the number of sorocarps occurred.

When a very few aggregations are formed early, as after a short initial dark period (Konijn and Raper, 1965), one would expect these centers to extend their spheres of attraction over large areas and subsequently to produce only a few and large sorocarps. However, this does not occur; instead many aggregations are formed although the increase in number occurs slowly. When all aggregations appear at about the same time, as after a long initial dark period, their size is generally larger and their number less.

The limited size of aggregations in the former populations may be due to a reduced acrasin secretion per cell as the size of the aggregate increases, and, after a certain acrasin concentration has been reached, additional cells entering the aggregate may not further increase the level of acrasin. This concentration may be sufficient to attract all cells within the aggregate's territory. This would conform with Shaffer's observation that acrasin sources of various sizes seem to secrete at the same concentration (Shaffer, 1957), and would support his assumption that the acrasin secretion per cell is inversely related to the size of the aggregate (Shaffer, 1962).

It is a common observation that streams of myxamoebae flowing into centers in darkness are longer than those in light. This may result in part from an increased stickiness of the cells in darkness that is reflected in somewhat longer streams, which in turn, since these also secrete acrasin, attract additional sensitive myxamoebae to further enlarge the aggregations. But this is only one of many ways in which light may act on aggregating myxamoebae.

Light may depress the formation of acrasin, or its precursors, or have a regulating action on the secretion of acrasin, *e.g.*, by changing the permeability of the cell membranes. If light affects the attraction of the cells it may do so by an

inactivation of acrasin, or by altering the sensitivity of the myxamoebae to it. That inactivation of acrasin, presumably enzymatic, does occur has been shown by Shaffer (1956). Another effect of increased inactivation of acrasin could be the occurrence of a steeper gradient, which would favor earlier aggregation. If inactivation of acrasin is enhanced by light, attraction should occur over a shorter distance in light, hence results in the formation of smaller aggregations and sorocarps. Smaller sorocarps were actually observed in plates incubated in the light, and a reduced attraction of responder cells in populations exposed to light was observed in an assay system in which attracting and responding cells were separated from each other by different distances at the time attraction occurred.

This work was made possible by research grants from the National Institutes of Health (CA 02119-09), U. S. Public Health Service, and the National Science Foundation (G-24953).

SUMMARY

1. Myxamoebae of *Dictyostelium discoideum*, NC-4(H), were pregrown in the dark on *Escherichia coli* or *Aerobacter aerogenes*, washed and deposited on non-nutrient agar. Populations incubated in constant light produced more and smaller aggregations and sorocarps than similar populations incubated in the dark. If populations were incubated in darkness for several hours and then transferred to light, the number of aggregations was reduced and the dimensions of these and the resulting sorocarps were correspondingly greater. The rate of increase in the number of aggregations was most rapid if the myxamoebae were exposed to a long initial dark period followed by light.

2. The chemotactic response of myxamoebae incubated in light or darkness was studied by depositing "attracting" and "responding" cells in separate small populations at predetermined distances from each other. The sphere of attraction by myxamoebae aggregating in light was found to extend over a shorter distance than that of cells aggregating in darkness. Among other possibilities, inactivation of the attracting substance(s) in the light may account for reduced attraction, hence result in smaller aggregations.

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PREDICTING DEVELOPMENT RATE OF COPEPOD EGGS¹

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This paper is part of a continuing study of intrinsic controls of growth, development and, implicitly, productivity of marine zooplankton. Copepods are particularly suitable for comparative studies of embryonic development rate because most species hatch at a morphologically equivalent first naupliar stage. The effect of temperature on size varies markedly among different geographical populations of the copepod *Pseudocalanus minutus* Krøyer, and it was assumed that embryonic development rate would vary likewise (McLaren, 1965a). However, after trips in the spring of 1965 to Woods Hole, Mass., Halifax, N. S., and Millport, Scotland, it was clear that embryonic development rate varied only slightly, and attention was directed to other species. A summer trip to Frobisher, N. W. T., was made particularly to secure data on a large-egged form of *Pseudocalanus* living in Ogac Lake, a warm, landlocked fiord off Frobisher Bay (McLaren, 1965a). Successful experiments at Frobisher on the large *Calanus glacialis* Jaschnov (see Grainger, 1961) were doubly useful because of published data on *C. finmarchicus* (Gunnerus) from Scotland and Norway (Marshall and Orr, 1953). A brief return to Halifax in April, 1966, added two more species, *Acartia clausi* Giesbrecht and *Tortanus discaudatus* (Thompson and Scott).

Although the number of forms studied is rather small, the results are published at this time because they seem to have some general and theoretical interest. More work will be done to confirm the results and hopefully to extend their predictive value to other developmental stages.

Use will be made of Bělehrádek's (1935, 1957) equation, in which rate of a metabolic function (here, development time D in days) is given by

$$D = a(T - \alpha)^b$$

where a , b and α are constants and T is the temperature. The empirical superiority of this equation and the conceptual meaning of its parameters have been discussed by McLaren (1963, 1965b). Briefly, the formula is the simplest of several equations describing the three ways in which monotonic responses to temperature may differ: a accounts for differences in mean slope, α for shifts on the temperature scale, and b depicts the degree of curvilinearity of the response quite adequately over the vital temperature range. This paper will show that the three parameters are related to separate biological properties as well. The equation is fitted by conversion to logarithms and successive approximation to that value of α having smallest sums of squares of deviations of observed from calculated development times.

¹ Supported by a grant from the National Research Council of Canada and a fellowship from the Canada Council.

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The author is grateful to the Woods Hole Oceanographic Institution, the Institute of Oceanography at Dalhousie University in Halifax, the Marine Station at Millport in Scotland, and the Department of Northern Affairs and National Resources at Frobisher in N.W.T. for working facilities and assistance. Drs. Carl M. Boyd, J. C. Carter, R. C. Conover, and Sheina M. Marshall, F.R.S., were especially helpful during the experimental programs. Mr. R. E. Banks, Naval Research Establishment, supplied temperature data from Halifax.

MATERIALS AND METHODS

The methods used were simple but at times onerous. Animals were captured by fine-mesh nets and samples kept cool during the return to the laboratory. Individual females were removed from the samples within an hour or two of capture with the aid of dissecting microscopes and eyedroppers. The females were kept in small groups in bottles at temperatures about as cool as the waters from which they were captured. During earlier series of experiments on *P. minutus*, concentrations of diatoms from net samples were added to the bottles in the hope of stimulating egg-laying. It was later found that eggs would be produced without the addition of food, provided the females came from phytoplankton-rich water. The bottles were observed regularly and free eggs (most species) or females carrying egg sacs (*Pseudocalanus*) were removed by eyedroppers as they appeared. These were placed in small vials with about 10 cc. of filtered sea water. Only a small proportion of females produced eggs, usually within a day or so of capture. Pertinent collection and experimental data are given on Table I.

The vials were kept at controlled temperatures until the eggs hatched. Accurate constant-temperature baths were available at Woods Hole, Halifax, and Millport. Baths in less well-controlled ambient temperatures of cold rooms were also available, and somewhat variable temperatures of around 2–3° C. were obtained in domestic-type refrigerators. Only a domestic refrigerator was available at Frobisher, and higher temperatures were maintained there by periodic additions of ice or warm water to large, covered, styrene-foam containers kept in the cool out-of-doors. These portable containers were also useful in completing experiments begun at Ogac Lake and finished at the townsite at Frobisher, a day's boat-trip from Ogac Lake. A temperature of 0° was easily obtained in ice-water baths kept just under the freezing compartments of domestic refrigerators. Because of the long time required for development, most of the experiments at 0° and some of those at higher temperatures were carried out on eggs produced by females brought by air to Montreal in vacuum bottles. A few experiments begun elsewhere were finished in Montreal—the vials kept at the appropriate temperatures in vacuum bottles during air transport. Clearly it was impossible to maintain rigidly constant temperatures under some of the above experimental conditions. However, temperatures were kept within narrow limits and observed frequently (at least every 3–4 hours), 24 hours a day. Since the effect of temperature is virtually linear over a reasonably narrow range, the graphically estimated mean temperatures may be taken as extremely accurate measures of the effective temperatures during development.

Experimental salinities were not quite the same in all localities, but differed markedly only in a second series of experiments on *Pseudocalanus* from Ogac Lake.

TABLE I

Mean development times of copepod eggs at different temperatures. Each experiment is a single egg sac (*Pseudocalanus*) or a batch of eggs produced more or less synchronously by one or more females (other spp.)

Species	Locality	Times of capture	Experimental salinity (‰)	No. of experiments	Mean experimental temperature (°C.)	Time of 50% hatch of viable eggs (days)	
						Mean $\pm 95\%$ f.l.	range
<i>Pseudocalanus minutus</i>	Woods Hole, Mass.	Jan. 27–Feb. 10, '65	31.8	7	0	10.89 $\pm$ 0.20	10.54–11.14
			31.8	3	3.18	6.61	6.49–6.75
			31.8	9	4.60	5.91 $\pm$ 0.09	4.43–4.70
			31.8	1	11.38	3.07	—
			31.8	3	13.13	2.78	2.69– 2.83
<i>Pseudocalanus minutus</i>	Halifax, N.S.	Mar. 19–29, '65	30.4	9	0	10.71 $\pm$ 0.22	10.56–11.14
			30.4	11	2.73	6.92 $\pm$ 0.09	6.71– 7.16
			30.4	4	5.35	5.05 $\pm$ 0.27	4.90– 5.31
			30.4	11	9.27	3.48 $\pm$ 0.04	3.38– 3.58
			30.4	14	12.01	2.90 $\pm$ 0.05	2.75– 3.00
<i>Pseudocalanus minutus</i>	Millport, Scotland	May 17–31, '65	30.4	4	0	11.15 $\pm$ 0.19	11.03–11.24
			30.4	2	2.65	7.50	7.42– 7.58
			30.4	1	3.14	7.15	—
			31.3	5	4.53	6.05 $\pm$ 0.14	5.92– 6.19
			31.3	5	6.64	4.84 $\pm$ 0.20	4.64– 4.94
			31.3	7	10.05	3.35 $\pm$ 0.13	3.20– 3.40
			31.3	5	12.95	2.75 $\pm$ 0.10	2.63– 2.79
<i>Pseudocalanus minutus</i>	Frobisher, N.W.T.	June 24–July 8, '65	32.3	7	0	10.44 $\pm$ 0.19	10.18–10.71
			32.3	9	2.49	7.50 $\pm$ 0.23	7.13– 8.00
			32.3	8	5.42	5.32 $\pm$ 0.13	5.19– 5.67
			32.3	5	7.90	4.14 $\pm$ 0.20	3.98– 4.40
			32.3	5	7.90	4.14 $\pm$ 0.20	3.98– 4.40
<i>Pseudocalanus minutus</i>	Ogac Lake, N.W.T.	July 18 & Aug. 3, '65	32.3	1	0	11.22	—
			25.7	3	0	10.86	10.61–11.21
			25.7	3	2.62	7.43	7.33– 7.62
			32.3	2	3.30	6.94	6.87– 7.00
			25.7	3	5.34	5.55	5.46– 5.67
			32.3	1	7.10	4.35	—
<i>Pseudocalanus</i> , large form	Ogac Lake, N.W.T.	July 18 & Aug. 3, '65	32.3	2	0	17.91	17.08–18.75
			25.7	1	0	17.27	—
			32.3	1	2.67	12.23	—
			25.7	1	3.31	10.46	—
			25.7	3	5.19	9.06	8.92– 9.30
<i>Calanus glacialis</i>	Frobisher, N.W.T.	July 8 '65	32.3	1	0	6.35	—
			32.3	1	2.60	4.30	—
			32.3	1	5.23	3.23	—
			32.3	1	7.92	2.61	—
<i>Acartia clausi</i>	Halifax, N.S.	April 5 & 7, '66	30.5	1	0	15.33	—
			30.5	3	4.88	5.98	5.88– 6.05
			30.5	3	10.80	2.78	2.69– 2.87
<i>Tortanus discaudatus</i>	Halifax, N.S.	April 5 & 7, '66	30.5	1	0	19.71	—
			30.5	3	4.85	8.15	8.00– 8.25
			30.5	3	10.80	3.93	3.82– 3.98

Much mortality had occurred during the first series of experiments at higher salinities. Since Ogac Lake is somewhat brackish, it was thought that filtered lake water would be more suitable, but mortality was still quite high. The possibility that salinity had an effect on development rate of eggs from Ogac Lake will be considered.

Not all eggs hatched, but those that did produced nauplii within a period of a few hours at most in a given vial. After eggs began hatching, experimental vials were observed frequently so that time of 50% hatch of viable eggs could be deter-

mined accurately. Above certain critical temperatures, which varied with locality, no eggs hatched. At lower temperatures, some batches of eggs failed partly or completely to hatch, and this sometimes appeared to be associated with bacterial or protozoan infestation. No antibiotics were used, but the sea water in some vials was changed when it appeared dirty. Arbitrarily, all experiments in which fewer than half the eggs proved viable are excluded from the following analyses, in view of the possibility that high mortality is accompanied by pathological or "unphysiological" retardation of development among the survivors.

TABLE II

Parameters of Bělehrádek's temperature function fitted to development time of copepod embryos

Species	Locality	Mean egg diameter ($\mu \pm 95\%$ f.l.)	Relative optical density (ratio $\pm 95\%$ f.l.)	Three constants fitted			<i>b</i> taken as -1.68	
				<i>a</i>	α	<i>b</i>	<i>a</i>	α
<i>Pseudocalanus minutus</i>	Woods Hole	127.4 $\pm$ 3.7	—	325	- 9.2	-1.53	552	-10.52
<i>P. minutus</i>	Halifax	121.6 $\pm$ 1.8	1.04 $\pm$ 0.10	159	- 6.3	-1.23	516	-10.20
<i>p. minutus</i>	Millport	123.6 $\pm$ 1.8	—	425	- 9.4	-1.62	536	- 9.99
<i>P. minutus</i>	Frobisher	130.4 $\pm$ 3.3	(taken as 1.00)	8433	-16.6	-2.39	572	-10.77
<i>P. minutus</i>	Ogac Lake	108.5 $\pm$ 2.3	0.95 $\pm$ 0.11	3296	-14.0	-2.16	543	-10.27
<i>Pseudocalanus</i> , large form	Ogac Lake	155.3 $\pm$ 2.6	1.04 $\pm$ 0.14	82	- 5.0	-0.95	908	-10.47
<i>Calanus glacialis</i>	Frobisher	178.6 $\pm$ 2.5	0.39 $\pm$ 0.03	44	- 6.1	-1.07	308	-11.23
<i>C. finmarchicus</i>	Tromsø	ca. 145	—	155	- 8.7	-1.57	231	- 9.72
<i>C. finmarchicus</i>	Millport	ca. 145	—	6	- 1.2	-0.65	204	- 9.43
<i>Artcartia clausi</i>	Halifax	79.4 $\pm$ 3.4	2.21 $\pm$ 0.35	1679	- 8.9	-2.15	322	- 6.02
<i>Tortanus discaudatus</i>	Halifax	102.4 $\pm$ 4.7	1.29 $\pm$ 0.18	2307	- 9.4	-2.12	477	- 6.55

Eggs were measured with optical micrometers at $\times 40$ or greater. Diameters given are of unpreserved eggs. Maximum and minimum diameters were averaged for near-spherical eggs, but the three appropriate diameters were taken of the flattened spheroid eggs of *T. discaudatus*.

A simple and perhaps rather crude method was used to determine optical density of formalin-preserved eggs, using a photomicrographic exposure meter (Photovolt Corp., New York, model 514-M). Eggs were placed on depression slides in clean formalin-sea water. Each egg was measured, then centered alone in the microscopic field at $\times 250$, in low, unfiltered illumination from a 6-volt wet-battery. The light cell was then applied to the photographic ocular and the meter deflection caused by moving the egg in and out of the center of the field was noted. Since the eggs are rather uniformly granular, it is assumed that no defraction problems were involved and that the meter deflections were a valid measure of the optical extinction caused by the volume of matter in the egg. Deflections were of the range of 5-20 units per egg; calibration is not exact, but one unit is of the order of 10^{-5} foot candles.

RESULTS

Effects of temperature

The experimental results are summarized on Table I. Clearly there are differences in temperature response among the various species. Development rate of *P. minutus* varies only slightly in different parts of its range. The differences are in some cases significant (compare, for example, the rate at 0° at Frobisher and Millport), but not nearly as great as expected from preliminary experiments on relative rate of development (McLaren, 1965a).

Bělehrádek's temperature functions have been fitted to all the data on Table I, and the resulting parameters are on Table II. For simplicity, mean development times were used, weighted by number of experiments at each temperature; the error in this procedure is probably small and unsystematic. Published data (Marshall and Orr, 1953) on development times of *C. finmarchicus* are also analyzed on Table II. The data were published as ranges in hours and were averaged for purposes of calculation.

Since the calculated values of α on Table II are generally well below the minimal experimental temperature (0°), the resolution of the function is very low, and the wide ranges of the three fitted parameters may be largely spurious. McLaren (1965b) suggested that the degree of curvilinearity of response to temperature (b) might be the same among related groups. The "real" value of b may be taken as the mean of estimates on Table II, each estimate weighted by the square root of number of determining experiments (excluding *C. finmarchicus* from Millport, for which number of experiments was not given). This mean may differ slightly from the true statistical mean, but the arguments that follow would not differ for any chosen value of b within the range on Table II, since α , $\log a$, and b are all linearly related. The new values of a and α are listed on Table II, and the empirical adequacy of the resulting curves is clear on Figure 1. Only two points deviate much from the curves; these points, at 2.7° and 3.3° for the large form of *Pseudocalanus*, represent individual experiments, whereas almost all other points are means. Assuming that b is in fact constant greatly increases the resolution of the function, even with inaccurate or biased data.

Taking b as 1.68 for all localities and species reduces the great range in values of α and regularizes them in a more logical way. Differences within species from various localities are then found to be very slight (range of 0.8° in *P. minutus* and 0.3° in *C. finmarchicus*), but differences between species are more marked. The most strictly arctic species, *C. glacialis*, has the lowest value of α . Unlike the other species, *A. clausi* and *T. discaudatus* do not extend to cold, northern regions, and this seems to be reflected in their higher values of α . At temperatures of -1°, which may be expected during the spring in arctic waters, *A. clausi* and *T. discaudatus* would take about three and four weeks simply to hatch their eggs.

Different values of α for embryonic development among thermal races of frogs (McLaren, 1956b) are significantly correlated with latitude or altitude, and therefore with environmental temperature. The *C. finmarchicus* studied by Marshall and Orr (1953) were said by these authors to be living at 2-3° and 6°, respectively, at Tromsø and Millport. The appropriate temperatures experienced by females of *P. minutus* can only be approximated. Temperatures for Halifax,

Woods Hole, and Millport are available as daily surface temperatures recorded by institutions at these localities. These are averaged for the periods during which females were captured. Temperatures were not taken at Frobisher or Ogac Lake during 1965. The waters below a few meters at Frobisher in late June and early July may be assumed to be at the winter minimum of *ca.* -1.7° . A published estimate (McLaren, 1965a, his Figure 2) of mean temperature in Ogac Lake ex-

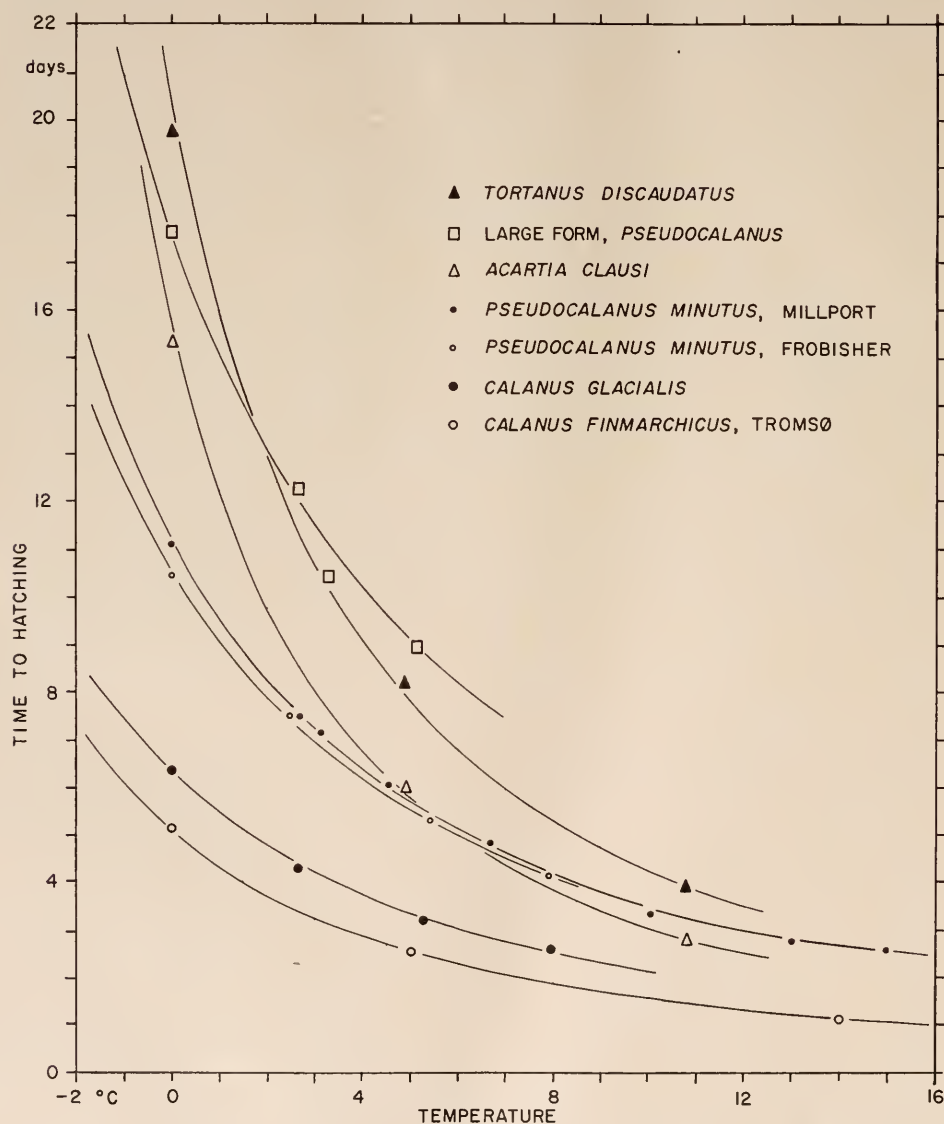


FIGURE 1. Bělehrádek's temperature functions fitted to development times of copepod embryos. The parameter b is taken as -1.68 for all curves, and the fitted values of α and a are given on Table II.

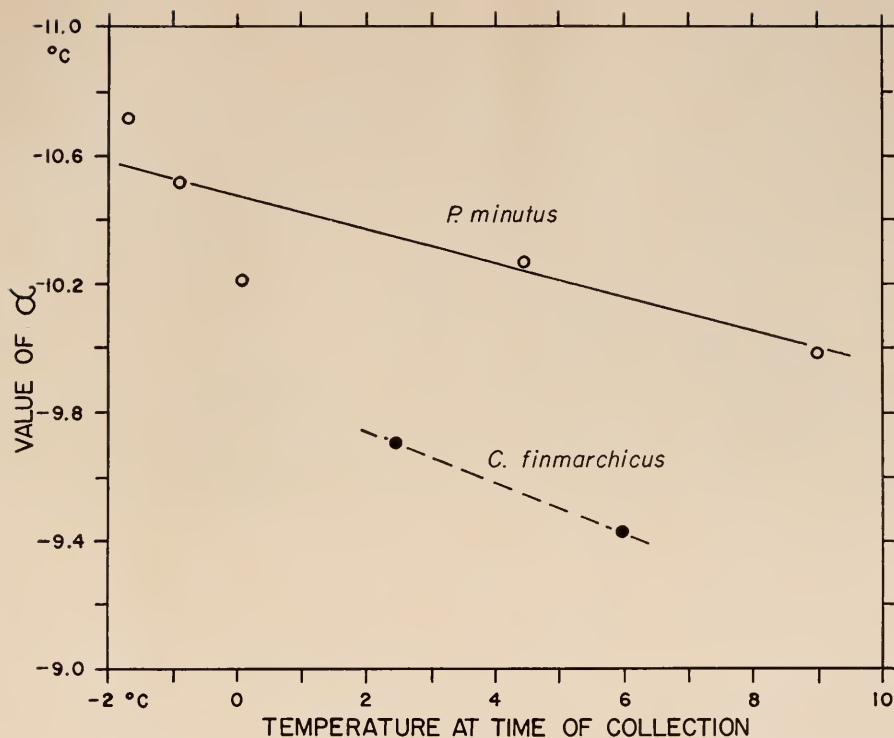


FIGURE 2. Relationship between the scale correction or "biological zero" α of Bělehrádek's function for development time of copepod embryos and estimated temperatures at the times egg-producing females were collected.

perienched by *P. minutus* maturing in early summer of 1957 may be used as a rough indication of temperatures at the same season in 1965. Values of α are plotted against these temperature estimates on Figure 2. The correlation for *P. minutus*, for which there are sufficient points to test, falls short of significant ($P \approx 0.10$). But the relationship is, as expected, positive for both species. The amount of intra-specific "adaptation" is very small, and is of about the same order as that found among thermal races of frogs (McLaren, 1965b).

Effects of egg size

Berrill (1935) found that development time of ascidian eggs was linearly related to egg diameter, provided the eggs were comparable in yoliness. McLaren (1965b) found that among thermal races of the frog *Rana pipiens*, α of Bělehrádek's function for embryonic development time was significantly correlated with egg diameter, at least within the United States. The same seems to be true among closely related forms of copepods (Fig. 3a). The correlation between α and egg diameter is significant ($0.05 > P > 0.01$) for *P. minutus*, excluding those from Ogac Lake. This is remarkable enough, considering the small number and re-

stricted range of values, and again seems to justify the assumption that b is the same for all populations.

In spite of their smaller size, the eggs of *P. minutus* from Ogac Lake developed at much the same rate as those from other areas. Differences in egg and body size of this species in the cold waters of Frobisher Bay and the warm waters of Ogac Lake are phenotypic (McLaren, 1965a). It may be that differences in egg size and its effects on a are not comparable to those occurring among the more widely

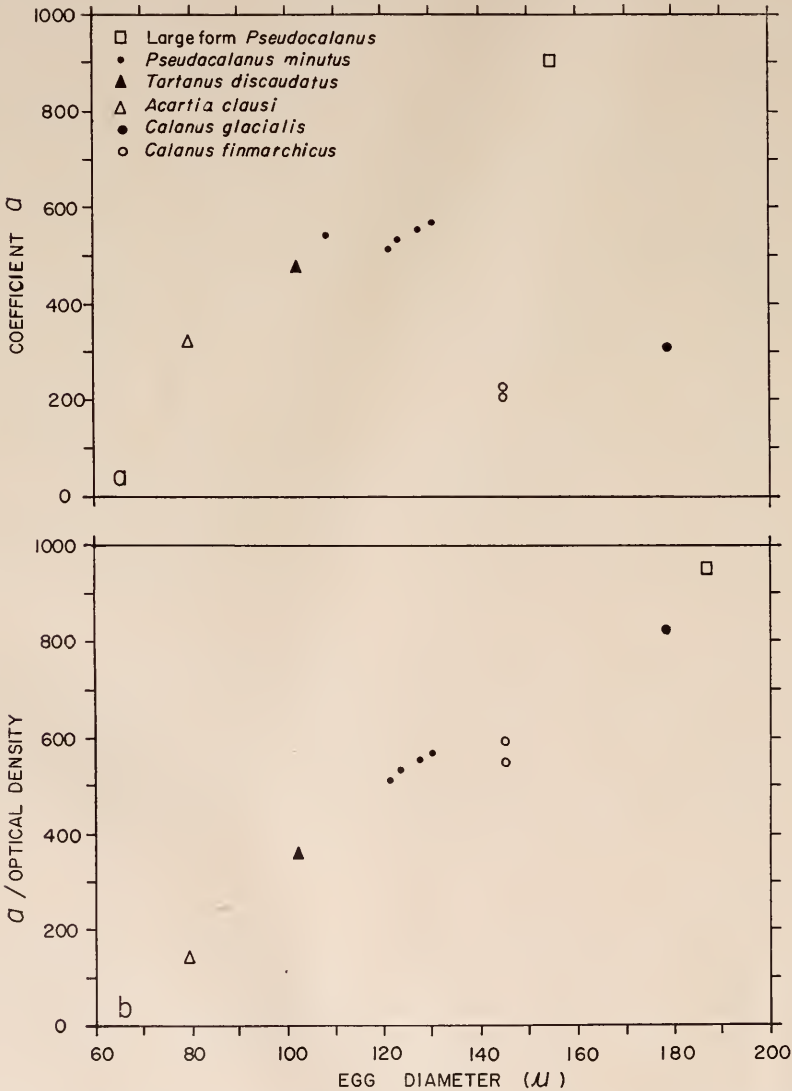


FIGURE 3. (a) Relationship between the proportionality coefficient a of Bělehrádek's temperature function for development time of copepod embryos and egg diameter. (b) The same after correction for yolk concentration (optical density). See text.

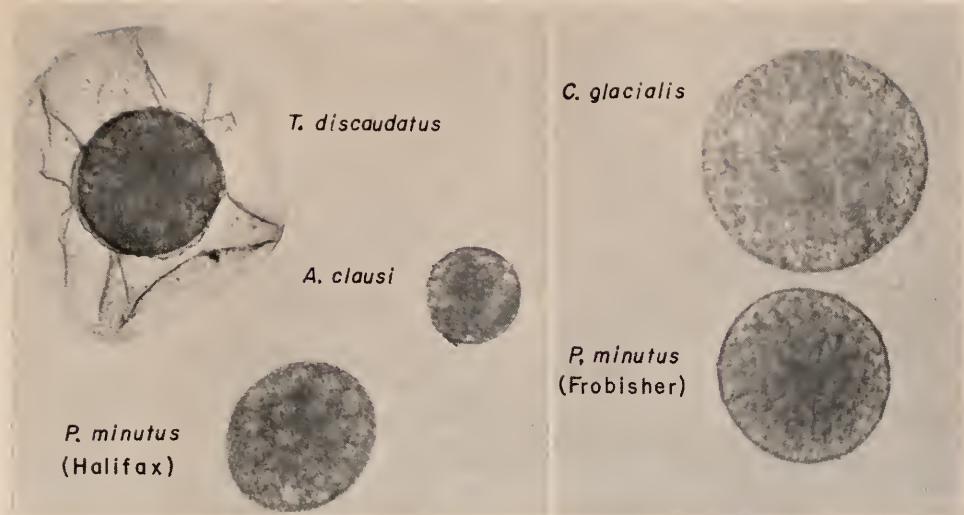


FIGURE 4. Representative copepod eggs to show differing optical density and size.

separated populations of the species. It is also possible that experimental salinities (see Materials and Methods) had an effect. Development was slower at 32.3‰ (Table I), which is abnormally high for the brackish population of Ogac Lake. There is evidence (Kinne, 1964) that growth rates, development rates, and sizes have optimal salinities. Whatever the explanation of the results from Ogac Lake, it seems possible to conclude that *P. minutus* and the large form of *Pseudocalanus* would have produced considerably larger eggs which would have developed only slightly more slowly than indicated, if they had been captured from the colder, more saline waters of Frobisher Bay.

The parameter a is not proportional to egg diameter. Assuming linearity, $a = 0$ at an egg size of about 35μ for marine *P. minutus*. For the large form of *Pseudocalanus* and *P. minutus* from Ogac Lake, the intercept is at about 54μ , and for *C. finmarchicus* and *glacialis*, at about 64μ . Since it is impossible that real eggs of these intercept sizes would develop infinitely rapidly, the assumed linear relationships are probably roughly tangential to shallow, concave functions, with origins at 0μ .

It is clear from Figure 3a that development times within species or among closely related forms may be partly predicted from egg size, but the same rule does not apply between distantly related species. For example, the eggs of *Calanus*, although much larger than those of *Pseudocalanus*, develop much more rapidly.

Effects of yolk concentration

Berrill (1935) concluded that development rate of ascidian eggs was retarded in proportion to the ratio of yolk to cytoplasm, although he made no quantitative measurements of yolk. All the copepod eggs studied here seem to be unpigmented, and are white to pale yellow in reflected light. Under transmitted light they differ

markedly in transparency (Fig. 4). It seems probable that transparency is related to concentration of yolk substances.

Newly produced eggs may be somewhat darker, and advanced embryos are more transparent, except for dark centers, which appear to be fat globules. Intermediate stages do not appear to vary systematically, and embryos ranging from few-celled to probable gastrula stages were chosen at random for measurements. Eggs of *Pseudocalanus*, *Calanus*, and *A. clausi* presented no special difficulties, although only a total of 11 eggs of the last species were available. Eggs of *T. discaudatus* are surrounded by a thick membrane, which is not completely transparent, and which often had a faint, reddish tinge. Thus, some of the extinction of transmitted light by these eggs is caused by the membrane. Fortunately, the complete membranes (Fig. 4) are cast by the hatching nauplii. A number were preserved for light measurements, and the results subtracted from measurements of intact eggs. Unfortunately, no corrections can be made for correlations of transparency of membranes and intact eggs.

The results are listed on Table II as mean extinctions per unit volumes of eggs, relative to the values for *P. minutus* from Frobisher Bay. Although they represent a 3-fold range in egg volumes, the eggs of the various populations and forms of *P. minutus* do not differ in optical density. This suggests that yolk concentration is the same among closely related forms, which also seems to be true of ascidians (Berrill, 1935). It also indicates the validity of the optical methods. In spite of large fiducial limits, due to small samples in most cases, there are marked and significant differences between species.

The exact relationship between yolk concentration and optical density cannot be proportional, but it may be nearly so, since cytoplasm is very transparent. The effect of yolk concentration on development time may be proportionate, but the effect of egg size is not (see above). Therefore the best way to express the possible effect of yolk is by dividing development time at a given temperature (or a among forms which differ in temperature characteristics) by yolk concentration (relative optical density). The results on Figure 3b clearly represent a marked regularization of the data on Figure 3a. Figure 3b also assumes, from arguments given above, that *P. minutus* is equivalent in size and development rate to the species in nearby Frobisher Bay, and that the large form of *Pseudocalanus* would be proportionately larger and slower in development if it occurred in Frobisher Bay.

The relationship on Figure 3b seems adequately described by

$$a = Y (6.51 D - 317)$$

where a is the proportionality coefficient of Bělehrádek's function in days, Y is the optical density relative to eggs of *Pseudocalanus*, and D is egg diameter in μ . Again, the real effect of D is probably not linear, and the relationship may take its origin from 0 μ .

DISCUSSION

Bělehrádek's temperature function clarifies analysis of the data. From the results, it should be possible to predict development rate of eggs of other species of copepods with a minimum of experimental data. Of perhaps more general interest is the further support for the conclusion of McLaren (1965b) that the

three conceptually separable parameters of Bělehrádek's function have separable biological meaning.

Temperature adaptation *per se* can be considered in relation to a single parameter, the scale correction α . This seems much simpler than discussion of " Q_{10} shifts," "translation," "rotation," and like terms, some of which are artifacts of the senilogarithmic plot and combine differences in slope (a), curvilinearity (b), and position on the Celsius scale (α).

Among closely related forms differences in a may be predicted from size alone. The large form of *Pseudocalanus*, with eggs and bodies about three times the volume of those of co-existing *P. minutus*, has the same chromosome number, but the chromosomes are much larger and contain several times as much DNA (McLaren, Woods, and Shea, 1966). A similar mechanism may account for the larger size of *C. glacialis*, which has the same chromosome number but larger eggs than *C. finmarchicus*. The inherent differences in size and development rate are perhaps related to DNA content in the manner suggested by Commoner (1964). However, a is not proportional to volume or DNA content, so that another form of control must be superimposed.

It is of interest to note that Berrill (1935) found a similar pattern of development among ascidian eggs. Among eggs larger than about 250 μ , development time was linearly related to egg diameter, with an intercept at about 125 μ . For smaller eggs between 100 and 170 μ the relationship was slightly curvilinear, with an apparent intercept at about 60 μ . This is comparable with copepod egg development, with an apparent intercept at about 50 μ (Fig. 3b). Berrill argued from proportionality (although this is not strictly true) of development time and egg diameter that control is imposed by surface-to-volume restrictions on CO_2 exchange of the whole embryo, and offered some experimental evidence for this. Recently Daykin (1965) applied mass transfer theory to the uptake of oxygen by fish eggs. It is not possible to define conditions applying to ascidian and copepod eggs, but representative solutions of Daykin's equations imply that the mass transfer coefficient is itself a positive function of egg diameter, with a negative second derivative. If the same form of coefficient applies to the outward transfer of CO_2 (which is much more likely than O_2 to be limiting), then the control suggested by Berrill would not result in strict proportionality between development time and egg diameter. Instead development time would increase curvilinearly with diameter among small eggs and more nearly linearly among larger eggs, more or less in the manner suggested for ascidian and copepod eggs. This suggests that Berrill's general ideas are sound, and that detailed analysis and prediction might be possible.

The influence of yolk does not seem explicable in energetic or biochemical terms. Although more yolky eggs take longer to hatch, many analyses of fat, protein, and energy have shown that very little is used by most developing embryos before they hatch (*e.g.*, Hayes, 1949). There may well be qualitative differences in the yolk of the several kinds of copepods, but its effect seems proportional to its crudely defined concentration. This seems to support Berrill's conclusion that yolk simply "dilutes" the metabolically active cytoplasm of the egg and embryo.

Bělehrádek (1935, 1957) believed he had a theoretical basis for his temperature function in observations that diffusion and viscosity, but not chemical reaction rates *in vitro*, are affected in a comparable, double logarithmic way. McLaren (1965b)

suggested that yolk, by affecting diffusion characteristics, might be involved in temperature adaptations. There is no evidence that α of copepod eggs is affected by concentration of yolk; the relationship (Table II) is positive but far from significant. If Bělehrádek's general ideas are correct, it may be that qualitative differences of yolk are involved, or that control resides in biophysical properties of the cytoplasm, rather than yolk.

SUMMARY

1. The development times to hatching of eggs of several kinds of copepods were studied at controlled temperatures. Data are analyzed from several geographical populations of *Pseudocalanus minutus*, a large form of *Pseudocalanus*, *Calanus finmarchicus*, *C. glacialis*, *Acartia clausi*, and *Tortanus discaudatus*.

2. Bělehrádek's temperature function, expressed as $D = a(T - \alpha)^b$, where D is development time, T the temperature, and α , a and b are constants, was fitted to the results. Assuming that b is the same (-1.68 , the mean of fitted values) for all species results in several regularities.

3. The scale correction or "biological zero" α varies little within species, but seems positively related to environmental temperature. *C. glacialis*, with the most northerly range, has the lowest value of α , and *A. clausi* and *T. discaudatus*, which are the most southerly, have highest values of α . Temperature adaptation *per se* may be considered in relation to this parameter alone.

4. The proportionality coefficient a varies significantly with egg diameter within species or between closely related species. Differences in a and egg size are related to differences in DNA content between *P. minutus* and the large form of *Pseudocalanus*, and the same may be true between other closely related forms.

5. The coefficient a is not exactly proportional to egg diameter or DNA content, but the relationship resembles predictions from mass transfer theory, and supports Berrill's (1935) belief that control is superimposed by surface/volume restrictions on CO_2 exchange by the whole embryo.

6. Differences in optical density of eggs are attributed to yolk concentration. The parameter α is proportionate to relative optical density, which supports Berrill's (1935) conclusion that yolk simply "dilutes" metabolically active cytoplasm. Yolkiness does not appear to affect other parameters, which it might do if it imposed restrictions on diffusion, as implied by the possible biophysical basis of Bělehrádek's temperature function.

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FACTORS INFLUENCING THE RESPONSE OF ISOLATED DOGFISH SKIN MELANOPHORES TO MELANOCYTE- STIMULATING HORMONE¹

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Although a good deal is known about the endocrinology of melanophores in elasmobranch fishes (Parker, 1948; Waring, 1963), little has been published regarding the hormonal responses of elasmobranch melanophores *in vitro*. The isolated skin of several species of dogfish pales in isotonic media and darkens upon the addition of pituitary extracts containing melanocyte-stimulating hormone (MSH), according to Lundstrom and Bard (1932) and Waring (1936, 1960). This fact correlates well with the established role of MSH in producing the darkening of elasmobranchs upon an illuminated black background. It also shows that dogfish melanophores are similar to frog melanophores in their *in vitro* behavior (Hogben and Winton, 1922). Although the molecular and cellular mechanism of the melanin-dispersing action of MSH is still unknown, MSH may produce melanin dispersion in amphibian melanophores by a sodium-dependent mechanism involving the uptake of water (Novales, 1959, 1962). Osmotic and ionic factors were also found to be of importance for the action of eye stalk hormone on the melanophores of the fiddler crab, *Uca pugnax*, by Fingerman, Miyawaki and Oguro (1963). In view of the relatively primitive nature of elasmobranch fishes, a study has been conducted of osmotic and ionic effects on the response of dogfish melanophores to MSH (Novales and Novales, 1966). It was hoped to learn more about the endocrine cellular physiology of elasmobranch melanophores and possibly to shed light on the evolution of melanophore control mechanisms. Although similarities exist between frog and dogfish melanophores, there are also differences, which could be of theoretical importance.

MATERIALS AND METHODS

The majority of experiments were performed with the isolated skin from a total of 12 female spiny dogfishes (*Squalus acanthias*). Some work was also done with three smooth dogfishes (*Mustelus canis*). Fishes were caught with hook and line and stored in sea water pens until use. They were kept overnight in an illuminated tank with a light background, resulting in some paling of their skin. The following day the fishes were decapitated. Skin, removed from the dorso-lateral trunk region, was placed in elasmobranch Ringer's solution (Cavanaugh, 1956), which had the following composition in g. per l. of distilled water: NaCl,

¹ This study was aided by grants from the National Science Foundation (G-24017) and the Office of Research Coordination, Northwestern University.

16.38; KCl, 0.89; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.47; NaHCO_3 , 0.38; dextrose, 1.00; urea, 21.6; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.07. Pieces of skin were mounted on aluminum frames of the type described by Shizume, Lerner and Fitzpatrick (1954) and immersed in 20 ml. of Ringer's solution in a 50-ml. beaker. The inside diameter of the upper elements of these frames had to be about one mm. larger than originally described, due to the greater thickness of dogfish skin. One fish furnished sufficient skin for 24 such frames, enough for a typical experiment of 6 experimental groups. The bath fluid was changed three times in a period of several hours, in order to produce complete blanching of the skin. At the end of this time the experiment was begun, utilizing the paled skin, with highly aggregated melanin in the melanophores. An initial reflectance reading was taken with a Photovolt photoelectric reflection meter, model No. 610, standardized to read 100 with the red filter and porcelain plate provided with the meter. The proper amount of MSH was then added to 20 ml. of Ringer or to the experimental medium. Preliminary time curves showed that full darkening requires 90 minutes. However, since virtually complete darkening had occurred within 60 minutes, the decrease in reflectance in galvanometer units (G.U.) occurring in one hr. (Δ) was used as the standard measure of darkening, as in previous work with frog skin (Novales, 1959). A standard MSH (S-MSH), a highly purified bovine β -MSH (β_b -MSH) and a synthetic α -MSH (syn. α -MSH) were used in the experiments. The S-MSH (Shizume, Lerner and Fitzpatrick, 1954) and β_b -MSH (Geschwind, Li and Barnafi, 1957) were obtained from C. H. Li; the syn. α -MSH (Hofmann and Yajima, 1962) was provided by K. Hofmann. All three hormones were prepared in lactose by the method of Novales, Novales, Zinner and Stoner (1962), to facilitate transporting, weighing and storage. The S-MSH had an activity of 10^9 units/g., when bioassayed by the method of Shizume, Lerner and Fitzpatrick (1954). The other MSH's showed comparable activity after bioassay at the sea shore. In view of the uniformity of the results obtained in the various experiments and with the three MSH's, the results from separate experiments were pooled. However, the majority of experiments were performed with the syn. α -MSH, which had a formyl group on the lysine at position 10.

Microscopic examination of melanophores showed that decrease in reflectance is accompanied by melanin dispersion in the dogfish melanophores. Cleared whole mounts were also prepared in some cases. Sodium analyses of the skin were performed by a similar method to that used for frog skin by Novales, Novales, Zinner and Stoner (1962). Skin was either removed directly from the fish or cut from the experimental frames after treatment. Wet weights were taken and dry weights obtained after drying overnight at 105°C . Samples were digested in one ml. of concentrated nitric acid in a boiling water bath and analyzed for sodium after dilution with distilled water, using a Coleman model 21 flame photometer.

RESULTS

The isolated skin of dogfish darkens when MSH is added to the medium bathing the skin. The log dose-response curves for the three MSH's acting on *Squalus* skin are given in Figure 1. The response to one unit/ml. of syn. α -MSH was significantly greater than the response to the same concentration of S-MSH ($p < 0.05 > 0.01$), but not significantly more than that to β_b -MSH. Since 100

units/ml. produced marked darkening, this concentration was chosen for further experiments on factors influencing the response. Urea was not required for the response. Thus, S-MSH at 10 units/ml. gave a Δ of 28 ± 2 G.U. (2) in urea-free Ringer. The numbers of skins are in parentheses.

The effects of a number of osmotic and ionic variables on the response of *Squalus* skin are given in Table I. There was a very uniform response in Ringer.

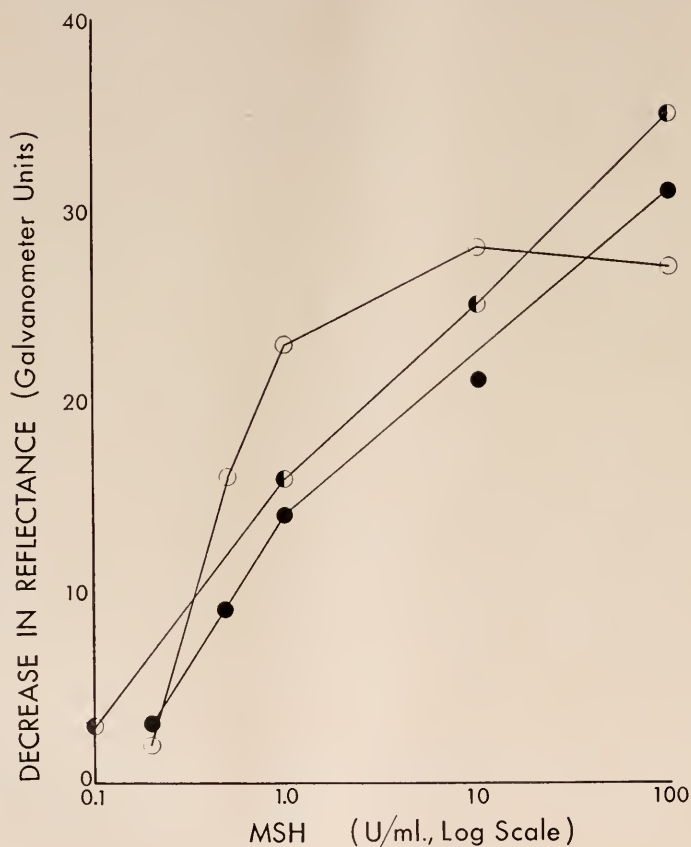


FIGURE 1. Log dose-response curves of paled *Squalus acanthias* skin melanophores to various types of MSH. Exposure was one hour. Points are means. (●) standard MSH, 24 skins; (○) syn. α -MSH, 36 skins; (◐) β -MSH, 14 skins.

The sodium content of skin freshly removed from the fish was 753 ± 49 (4) mM/kg. dry wt., which is not significantly different from that found after maintenance in Ringer for hours, as given in Table I. This testifies to the good physiological qualities of the Ringer's solution used here.

Bathing in hypotonic Ringer containing $\frac{1}{4}$ of normal Ringer sodium produced slight darkening, as seen in Table I. MSH produced a normal total darkening in the hypotonic Ringer (Table I). Hypotonic Ringer of $\frac{1}{2}$ and $\frac{3}{4}$ normal Ringer sodium produced no significant darkening alone, showing the resistance of dogfish

melanophores to the darkening effect of hypotonicity. Very dilute Ringer with $\frac{1}{10}$ normal sodium produced a marked darkening of 18 ± 1 (2) G.U. However, MSH failed to produce any more darkening than this, showing the unphysiological nature of this medium. Melanin was dispersed in melanophores treated with the hypotonic Ringer. However, distilled water produced a "cloudy" partially dispersed condition, suggesting that possibly osmotic cytolysis of the melanophores had occurred. The sodium content of skin treated with $\frac{1}{10} \times$ Ringer was 223 ± 16 (5) mM/kg., a marked reduction below that of fresh skin or skin treated with normal Ringer's solution.

Although hypertonicity by itself has no effect on the melanophores, Ringer's solution made hypertonic with sucrose (Table I) blocks the action of MSH. MSH at the lower concentration of 10 units/ml. was also inhibited in the hypertonic

TABLE I
Factors influencing the response of Squalus melanophores to MSH

Medium	1-Hr. darkening (Δ , in G.U.)*		Skin Na after treatment (mM/kg. dry wt.)
	MSH Absent	MSH Present†	
Elasmobranch Ringer	1 ± 0.4 (9)**	29 ± 1 (25)	713 ± 50 (5)
Hypotonic Ringer ($\frac{1}{4} \times$)	8 ± 2 (8)	29 ± 2 (8)	—
Hypertonic Ringer ($2 \times$)	1 ± 1 (7)	11 ± 1 (8)	—
Na-free Ringer (lithium)	1 ± 1 (6)	27 ± 3 (4)	130 ± 10 (4)
Na-free Ringer (choline)	4 ± 1 (8)	26 ± 3 (12)	—
Isotonic†† NaCl (313 mM)	$+1 \pm 1$ (3)	23 ± 3 (4)	—
Isotonic sucrose (625 mM)	6 ± 1 (5)	9 ± 1 (8)	—
Isotonic KCl (313 mM)	8 ± 0.3 (4)	27 ± 2 (4)	146 ± 1 (4)
Isotonic MgCl ₂ (208 mM)	10 ± 2 (4)	21 ± 2 (4)	146 ± 14 (4)
Isotonic CaCl ₂ (208 mM)	0 ± 1 (5)	0 ± 0.3 (8)	—

* Decrease in reflectance in galvanometer units.

† MSH concentration of 100 units/ml.

** Figures are means $\pm$ standard errors; numbers of skins in parentheses.

†† Isotonic solutions contained urea and dextrose as in Ringer.

medium. Ringer made hypertonic with NaCl to give a final NaCl concentration of 0.56 M completely inhibited MSH action, indicating that a high sodium content is toxic to the melanophores. Furthermore, MSH was markedly inhibited in sea water, which was calculated to be about $1.6 \times$ isotonicity. The hypertonic inhibition was reversible, for skins darkened with MSH upon transfer to normal Ringer after having been in a hypertonic medium.

The effect of replacement of sodium by a variety of other substances is also shown in Table I. Sodium-free Ringer was prepared with LiCl replacing the NaCl, and potassium salts the other sodium salts. MSH was fully active in the lithium Ringer, even though this medium produced an 82% reduction in the sodium content of the skin. MSH was also active in sodium-free choline Ringer (Table I). After these experiments showing that sodium can be replaced in normal Ringer, a simplified type of medium was used, consisting of a single chloride as well as urea and dextrose as in normal Ringer. An isotonic NaCl solution

TABLE II
Factors influencing the response of *Mustelus melanophores* to MSH

Medium	1-Hr. darkening (Δ , in G.U.)*	
	MSH absent	MSH present†
Elasmobranch Ringer	2 ± 1 (4)**	41 ± 4 (8)
Hypotonic Ringer ($\frac{1}{4} \times$)	$+1 \pm 1$ (4)	27 ± 3 (5)
Hypertonic Ringer ($2 \times$)	6 ± 2 (4)	17 ± 2 (7)
Na-free Ringer (choline)	2 ± 2 (4)	32 ± 2 (8)

* Decrease in reflectance in galvanometer units.

† MSH concentration of 100 units/ml.

** Figures are means $\pm$ standard errors; numbers of skins in parentheses.

of this type fully supported the response to MSH (Table I). However, there was a two-thirds reduction in the amount of response in an isotonic sucrose medium, showing the need for cations in the response. MSH action was also inhibited in sodium-free Ringer prepared with sucrose, for 10 units/ml. gave a darkening of only 11 ± 2 (4), much lower than the response in normal Ringer, which was about twice as great (Fig. 1). Surprisingly, a strong response was obtained in the isotonic KCl and $MgCl_2$ media (Table I). Either of these solutions alone produced a substantial darkening, probably as a result of their acidity, for their pH values were 6.3 and 6.0, respectively. Shizume, Lerner and Fitzpatrick (1954) found that solutions below a pH of 6.5 will darken frog skin. Both media were effective in reducing the sodium content of dogfish skin. Finally, the $CaCl_2$ medium completely failed to support darkening. This failure was not reversible, indicating that a high calcium concentration is probably toxic to the melanophores.

Similar results were obtained with the skin of the smooth dogfish, *M. canis*, as shown in Table II. *Mustelus* skin gave a greater reflectometric change with MSH than did *Squalus* skin. However, ($\frac{1}{4} \times$) hypotonic Ringer had no significant darkening alone and the response to MSH was significantly lower in this medium than in normal Ringer ($P = 0.01$). Thus, *Mustelus* is slightly more resistant to

TABLE III
The effect of caffeine* on dogfish skin

Medium	1-Hr. darkening† (Δ , in G.U.)	
	<i>S. acanthias</i>	<i>M. canis</i>
Elasmobranch Ringer	19 ± 2 (6)**	16 ± 6 (2)
Na-free Ringer (choline)	26 ± 3 (4)	28 ± 1 (2)
Isotonic†† sucrose (625 mM)	15 ± 1 (4)	—
Isotonic $CaCl_2$ (208 mM)	0 (4)	—

* Caffeine concentration of 0.01 M.

† Decrease in reflectance in galvanometer units.

** Figures are means $\pm$ standard errors; numbers of skins in parentheses.

†† Isotonic solutions contained urea and dextrose as in Ringer.

hypotonic darkening than *Squalus*. In hypertonic Ringer there was about a 60% reduction in the response to MSH. However, in sodium-free choline Ringer the response to MSH was not significantly different than in normal Ringer; thus sodium can be replaced in the case of *Mustelus* also.

Experiments with caffeine shed light on the specificity of the above effects on MSH darkening. This agent darkened the skin of both species at 0.01 M, as seen in Table III. Moreover, in sodium-free Ringer, there was a greater darkening, although the difference is not statistically significant. This difference was not seen in the isotonic sucrose, however. No response was obtained in the isotonic CaCl_2 , further demonstrating the toxic effect of this solution on the melanophores.

DISCUSSION

The present study extends earlier results with the isolated skin of *Mustelus canis* and *Scyllium canicula* to *Squalus acanthias*. Lundstrom and Bard (1932) first showed that hypophysectomy causes paling of the dogfish. Melanophores in isolated skin of *M. canis* contracted their pigment in dilute sea water. Melanin dispersion occurred with mammalian or *Mustelus* posterior lobe extract. Waring (1936) obtained similar results with *S. canicula* skin. Because he found that melanin aggregation occurs slowly in dilute sea water, we allowed at least two hours of Ringer rinses to produce maximum paling. Waring (1960) also obtained a graded response to increasing amounts of hormone, as we did in Figure 1. He obtained a good response in Young's elasmobranch saline (dilute sea water and urea) and we did also in our earliest experiments. However, we adopted the elasmobranch Ringer, because of the ease of modifying its composition.

The present results also support the conclusion of Parker (1936) that darkening is brought about in *S. acanthias* by a pituitary hormone and paling by the absence of this hormone, since isolated skin pales maximally when rinsed and darkens maximally upon addition of MSH. Highly purified mammalian hormones act on dogfish melanophores, just as they do on frog melanophores, in confirmation of earlier work showing that a variety of MSH-containing preparations disperse melanin in elasmobranch melanophores (Pickford and Atz, 1957).

The dispensability of urea in the response of *Squalus* melanophores is another indication that the high blood urea of elasmobranchs probably functions solely to maintain osmotic balance (Smith, 1936). Frédéricq (1922) found that the heart of the dogfish *Scyllium catulus* continued to beat in a urea-free salt solution. *Squalus* melanophores are also capable of responding to MSH in the absence of urea.

Waring (1936) observed that dogfish color changes are largely due to changes in the dermal melanophores. The gross and microscopic aspects of color change in *S. acanthias* are illustrated in Waring and Landgrebe (1950). Dogfish color changes require days to occur *in vivo* (Parker, 1948), but only a few hours *in vitro*. Thus, the greater length of time required *in vivo* must be due to the slowness of the control of MSH secretion, rather than to any slowness in the response of melanophores to MSH.

The darkening effect of hypotonic media shows that water entry is capable of producing melanin dispersion in *Squalus* melanophores. Furthermore, *Squalus* melanophores are less sensitive than frog melanophores to the dispersing effect of

hypotonic media. Thus, whereas $\frac{1}{4} \times$ Ringer darkens frog skin about 50% as much as MSH (Novales, 1959), it only darkens *Squalus* skin 28% as much as MSH (Table I). Hypotonic media also disperse melanin in the melanophores of frog (Shizume, Lerner and Fitzpatrick, 1954), bony fish (Spaeth, 1913), fiddler crabs (Fingerman, Miyawaki and Oguro, 1963), and salamanders in tissue culture (Novales and Novales, 1965). This effect has been used to support the view that MSH action may involve the uptake of water by the melanophore (Novales, 1959, 1962). Another way of demonstrating the role of water movement is to show an inhibitory effect of hypertonicity on the response to MSH. This was done in both dogfish species studied (Tables I, II). Hypertonicity inhibits melanin dispersion in frog, fiddler crab and salamander melanophores (*loc. cit.*). However, since dispersion produced by drugs as well as aggregation are inhibited (Novales, 1959), this effect does not establish a role of water entry in MSH action, since the inhibition is not specific to the effect of MSH.

The present study has also shown that dogfish melanophores are able to respond to MSH in sodium-free media. Sodium can be replaced by lithium, choline, potassium or magnesium ions in the response of *Squalus* melanophores, but sucrose or calcium fail to replace sodium (Table I). Thus, a cation must be present for the response to occur. The toxicity of calcium is shown by the failure of caffeine to act in a sodium-free calcium medium, whereas it is effective in a sucrose medium, in contrast to MSH (Table III). The cation requirements for dogfish melanophore responses are thus clearly different from those for frog melanophores. Whereas sodium can be replaced by a variety of other cations in the dogfish response, the requirement for sodium is absolute in the case of the frog response to MSH (Novales, Novales, Zinner and Stoner, 1962). It is unlikely that sufficient sodium was present in the dogfish skin to permit a response to MSH, if sodium were required. About 80% of skin sodium was removed by the sodium-free media (Table I). Since frog skin fails to respond when 90% of its skin sodium is removed (*loc. cit.*), it is unlikely that dogfish skin would respond with 80% of its sodium gone, if sodium were required for the response. Of the ions capable of replacing sodium in the response of *Squalus* skin to MSH, neither lithium, choline or potassium is capable of replacing sodium in the response of frog skin melanophores. The present results also recall those obtained with the fiddler crab by Fingerman, Miyawaki and Oguro (1963). They found that sodium can be replaced by other monovalent cations such as potassium or lithium in the response of melanophores to eyestalk hormone, but divalent cations such as magnesium and calcium fail to replace sodium. Thus, this system differs in that magnesium is able to replace sodium in the dogfish response.

Sodium can be replaced by other cations in a variety of other excitable systems, such as nerve and muscle (Spyropoulos and Tasaki, 1960). However, this does not necessarily mean that sodium ions are not involved in the responses *in vivo*. Thus, sodium has so far been irreplaceable in the response of *Rana pipiens* melanophores to MSH, for virtually all the cations capable of replacing sodium in nerve and muscle excitation have been tried and failed (Novales, 1959; Wright and Lerner, 1960; Novales, Novales, Zinner and Stoner, 1962). On the other hand, the present study has shown that *Squalus* melanophores will respond to MSH when sodium is replaced by a variety of cations. However, these results do not

mean that sodium is not involved in the *Squalus* response *in vivo*. Sodium probably is involved. It merely means that the specificity of the cation-requiring system is broad in *Squalus* but extremely narrow in *Rana*. This difference in specificity could be a reflection of the more primitive nature of the elasmobranch fish when compared with the anuran amphibian. The sodium-requiring process involved in MSH action has apparently increased in its specificity to sodium during evolution. Another possibility is that modern elasmobranchs have lost the high specificity of the sodium requirement possessed by their ancestors. However, this is an unlikely explanation which would be difficult to prove. In view of the phylogenetic position of bony fishes between the cartilaginous fishes and amphibians, information is needed regarding the cation requirements for MSH action in teleost fishes. Information about the requirements in cyclostome fishes would also be of interest, since they are more primitive than elasmobranchs.

The authors are indebted to Dr. C. H. Li of the University of California, Berkeley, for providing the natural MSH. Dr. Klaus Hofmann of the University of Pittsburgh kindly provided the synthetic MSH. Dr. Lois TeWinkel of Smith College aided us greatly in our early fishing expeditions and the Staff of the Marine Biological Laboratory provided facilities and numerous essential services.

SUMMARY

1. The log dose-response curves to standard MSH, bovine β -MSH, and synthetic α -MSH were obtained for the melanophores of isolated *Squalus acanthias* skin, using a reflectometric technique.

2. Urea is not required for the response of *Squalus* melanophores to MSH, further supporting the view that urea is required solely for maintaining the osmotic balance of elasmobranchs.

3. Hypotonic Ringer ($\frac{1}{10} \times$) produces marked darkening of *Squalus* skin, indicating that water entry can cause melanin dispersion.

4. Hypertonic Ringer ($2 \times$) inhibits MSH action on *Squalus* melanophores, indicating that water entry may occur during MSH action.

5. MSH can act on *Squalus* or *Mustelus* melanophores in the absence of sodium and lithium; choline, potassium and magnesium are all capable of replacing sodium in the response of *Squalus* melanophores.

6. MSH action is reduced in a sodium-free sucrose medium; thus there is a cation requirement for MSH action on *Squalus* melanophores.

7. Either there is no sodium requirement for MSH action on the dogfishes studied, or the specificity of the sodium requirement for sodium in the dogfish is much lower than in the frog.

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DURATION AND FREQUENCY OF WING BEAT IN THE AGING HOUSE FLY, *MUSCA DOMESTICA* L.¹

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In a long-range study of aging in the house fly, Rockstein and his co-workers (Rockstein, 1956, 1957; Rockstein and Brandt, 1963; Rockstein and Gutfreund, 1961) have previously noted that abrading and ultimate loss of wings, especially in the male house fly, is preceded and then accompanied by the failure or decline of specific intracellular biochemical components (enzymes, coenzymes, etc.) of the thoracic flight muscle, which are directly or indirectly concerned with the energizing of the contraction of flight muscle. Corresponding microanatomical changes were similarly reported by Rockstein and Bhatnagar (1965) in describing the age-related distribution of number and size of giant mitochondria of maturing and senescent male and female house flies.

The study on which this report is based was undertaken in order to establish more precisely the age-related, quantitative changes in flight ability, *i.e.*, the wing beat frequency and duration of flight, for male and female house flies from emergence to senility.

MATERIALS AND METHODS

A long-inbred (NAIDM) strain of *Musca domestica* L., maintained in our laboratory at 80° F. and 45% R.H., was used in this study. The experimental population was obtained by allowing 4- to 5-day-old females to lay eggs on a standardized artificial medium described earlier (Rockstein, 1957). From the time of emergence and during the course of the entire experiment, all flies were allowed to feed *ad libitum* on sucrose, twice daily, for a period of one hour each feeding; such flies were considered to be fully satiated as far as their carbohydrate requirements were concerned. A continuous supply of water was provided throughout the period of study.

For the study of wing beat frequency (WBF), flies of known age, immediately after having been previously fed on sucrose for an hour, were anesthetized under carbon dioxide, sexed, and mounted (attached individually in the dorsal midthoracic region with Duco® cement) onto thin, inverted "J"-shaped copper wire supports, which have been set in fine holes drilled in a wooden block 6" × 1.5". Removal of tarsi or of any substratum, essential for the initiation and sustenance of flight in the case of *Phormia* (Friedman, 1959; Clegg and Evans, 1961) or of *Drosophila* (Williams *et al.*, 1943), was found to be quite unnecessary for initiating or maintaining flight in the house fly, which normally flies spontaneously when so sup-

¹ Supported in part by funds from the United States Public Health Service, Research Grant No. HD 00571 from the National Institute of Child Health and Human Development.

ported. This total process of anesthetizing and mounting takes less than 5 minutes and, if normal, the flies begin to fly spontaneously within two to three minutes after having been mounted and after recovery from CO₂ anesthesia. In fact, those flies which did not fly spontaneously would not do so even upon stimulation of their tarsi or of the ventral surfaces of the abdomen. All phases of the experiments were conducted under constant conditions of temperature, humidity and light as previously described by Rockstein (1956).

Wing beat frequency was measured by means of a Xenon Stroboscope (Cenco), with the mounted flies placed about 6 inches from the emission tube and observations made at 5-minute intervals and expressed as a percentage of the initial rate (Clegg and Evans, 1961). In each case, such observations were continued until 90% or more of the flies could no longer fly. Such experiments were repeated over a period of four generations.

TABLE I

Wing beat frequency and duration of flight of the female house fly as a function of age

Age (days)	No. of specimens	Initial WBF $\pm$ S.E.	Flight duration (in minutes)
1	16	846.3 $\pm$ 109	500
2	17	958.4 $\pm$ 151	475
4	22	959.8 $\pm$ 129	455
5	10	983.8 $\pm$ 189	465
6	25	985.2 $\pm$ 136	470
7	29	991.8 $\pm$ 111	440
10	28	996.5 $\pm$ 164	398
15	33	986.9 $\pm$ 166	225
19	19	985.7 $\pm$ 207	105
22	22	985.5 $\pm$ 150	110

RESULTS AND DISCUSSION

Females

Table I shows that the average WBF of a one-day-old female fly is 8500 beats per minute (bpm) and that this increases to 9600 by the second day and reaches a maximum of approximately 10,000 bpm by the seventh day; thereafter, there is very little change in WBF up to the third week, with perhaps a slight (if at all significant) diminution in WBF at the beginning of the third week and into the last day of adult life on which such observations could be made.

The time course of WBF of a one-day-old female is represented in Figure 1. For the first 155 minutes, the flies fly at a maximum WBF and thereafter they show a gradual decline. Even after 500 minutes of continuous flight, however, the flies show speeds as high as 90% of the initial WBF. Two-day-old female flies, with a WBF of 9600 beats per minute, show a constant WBF flight pattern up to about 250 minutes (Fig. 2), when all of the flies fly at about 95% of the initial WBF. However, during the last 200 minutes of flight, the WBF of such two-day-old females declines gradually to a low of less than 80% of the original. For female flies up to 10 days of age, the WBF and the time course of flight patterns are quite similar to those of younger, two-day-old females. However, only

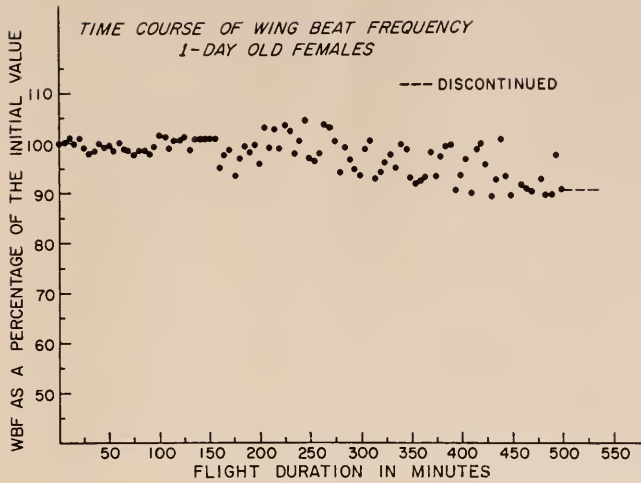


FIGURE 1. Time course of wing beat frequency in one-day-old female house flies.

after 12 days, when the WBF is still at its peak level, does the time course of flight change appreciably (Fig. 3), with a more rapid decline within 310 minutes to a WBF minimum. Finally, in 19-day-old females (Fig. 4), there is a rapid decline to a minimum in WBF in a little over 100 minutes.

Thus, the most conspicuous manifestation of senescence in flight function is the inability of very old flies to sustain flight for any extended periods of time. To recapitulate (as can be seen from Table I), there is a slow, steady, day-to-day decline in the duration of flight, so that by the third week, the ability of the female house fly to maintain flight for long periods of time is reduced markedly from 500 minutes in one-day-old to 110 minutes in 22-day-old female flies. This represents a decline in the ability of aging females to maintain flight to about 50% of the

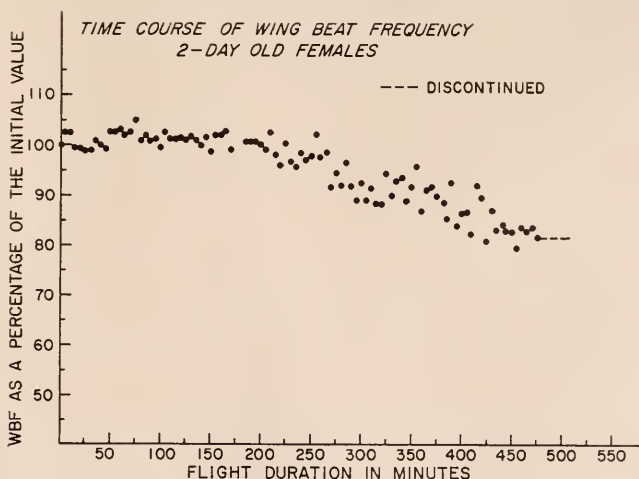


FIGURE 2. Time course of wing beat frequency in two-day-old female house flies.

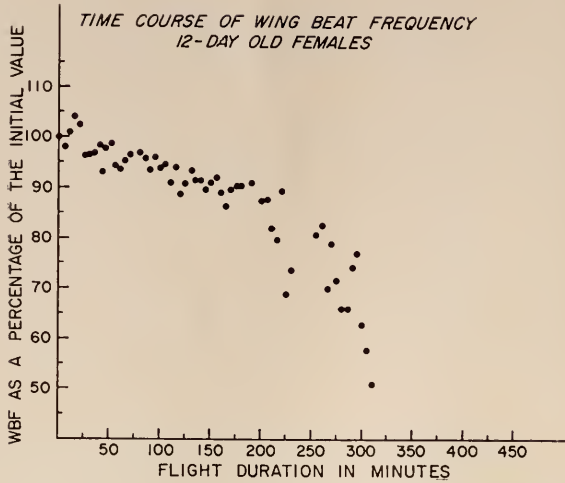


FIGURE 3. Time course of wing beat frequency in 12-day-old female house flies.

maximum level of flight duration at two weeks of age and to 20% of the initial maximum by the third week of adult life.

Males

As regards age-related failure in flight ability of the senescent male house fly, it should be emphasized that this particular study was carried out under special conditions of maintenance of all adult flies (*i.e.*, feeding them sucrose alone). Under such conditions, the rate of dying is much accelerated, *i.e.*, 60% of the males were dead by the 7th day, and of the remaining males at this age, only a few retained their wings (see Rockstein, 1956).

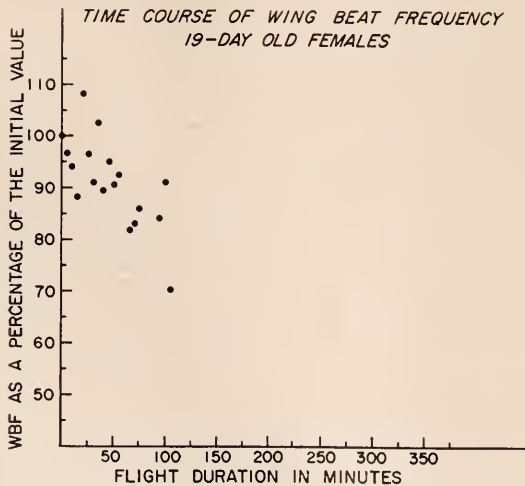


FIGURE 4. Time course of wing beat frequency in 19-day-old female house flies.

TABLE II

Wing beat frequency and duration of flight of the male house fly as a function of age

Age (days)	No. of specimens	Initial WBF $\pm$ S.E.	Flight duration (in minutes)
1	19	8639 $\pm$ 141	420
2	21	9328 $\pm$ 109	365
4	28	9710 $\pm$ 193	265
5	13	9698 $\pm$ 183	220
6	26	9733 $\pm$ 123	180
7	17	9801 $\pm$ 138	135
8	9	9821 $\pm$ 163	125
9	8	9700 $\pm$ 183	63

Moreover, under the above-mentioned conditions, 60% to 70% of the male population in the fly colony died within five to six days of eclosion and, out of the 30% to 40% remaining survivors which could be studied for flight ability, only a few retained intact wings, even as early as the end of the first week of imaginal life.

From Table II, it can be seen that one-day-old males fly at about 8600 wing beats per minute, which compares favorably with that for females of the same age (see Table I, above). This increases to about 9300 wing beats per minute during the following 24 hours. Maximum WBF of about 9700 to 9800 bpm is reached within four days after emergence and this remains unchanged through the ninth day of adult life, following which time no winged flies were available.

Figure 5 shows WBF as a function of flight duration in the one-day-old male house fly. The time pattern of the WBF of such young flies appears to be very similar to that of two-day-old female house flies (Fig. 2), *i.e.*, the WBF declines by about 18% by the end of 420 minutes, at which time the majority of the flies have stopped flying.

By the fifth day after emergence, male flies show a sharp decline in their WBF (Fig. 6) with time, *i.e.*, from the onset to the termination of flight, with the majority

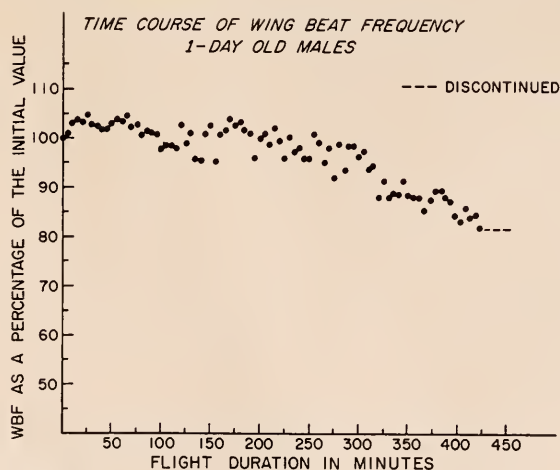


FIGURE 5. Time course of wing beat frequency in one-day-old male house flies.

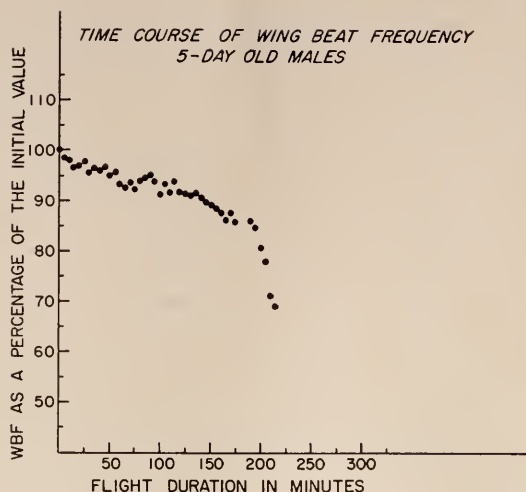


FIGURE 6. Time course of wing beat frequency in 5-day-old male house flies.

of flies showing a decline of about 30% within 215 minutes. The rapidity of such decline becomes more pronounced with age, so that seven-day-old males (Fig. 7) also show a decline by about 30% of WBF, but at 130 minutes after the initiation of flight.

It is quite apparent that the detailed data from these experiments confirm the more gross manifestations of senescence in flight ability, *i.e.*, the gradually increasing rate of failure of wing retention (male flies especially) previously observed by Rockstein (1956) and by Rockstein and Brandt (1963).

It would therefore appear from these data that the onset of decline in the motor function of flight in this holometabolous species clearly begins, as might be expected,

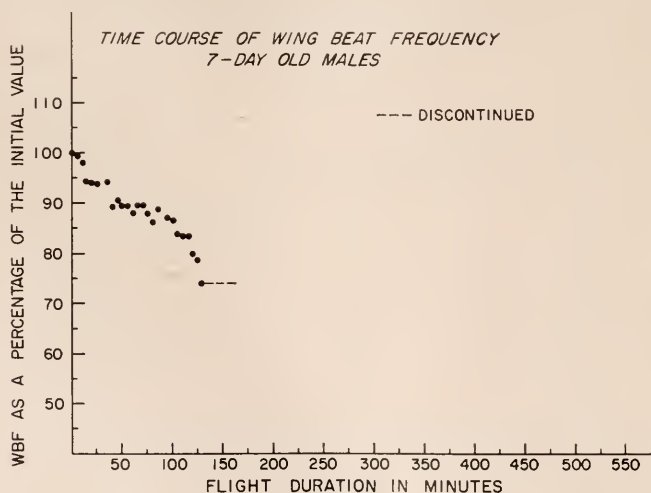


FIGURE 7. Time course of wing beat frequency in 7-day-old male house flies.

shortly after emergence, when the insect is fully mature and all of its tissues are essentially post-mitotic (with the exception of sexual maturation).

However, Williams *et al.* (1943) found this not to be true in *Drosophila funebris*, where both flight duration and wing beat frequency reach a peak by about 7 days and then drop off rapidly to a minimum by 35 days of adult life. However, no distinction as to relative flight ability of male and female *Drosophila* (either as regards WBF or duration of flight) was made by these authors, a distinction which both our present study and others have indicated may be a significant factor in all species studied.

In the two other important reports in which flight ability was studied (both in *Phormia regina*), unfortunately only WBF, with no distinction as to sex, was determined for aging adult blow flies by Levenbook and Williams (1956) and without regard to sex or age by Clegg and Evans (1961). It is therefore difficult to attempt comparisons between data for this present study and those in the relatively few past studies of this kind.

As for the trends observed in Tables I and II for wing beat frequency, values of about 10,000 beats per minute in mature males and female house flies resemble closely those reported both by Levenbook and Williams (1956), and by Clegg and Evans (1961) for *Phormia regina*. It is clear, however, that the parameter of duration of flight is much more significant, from the standpoint of aging, and that this parameter must also be measured separately for male and female adults, at least as far as the common house fly, *Musca domestica*, is concerned.

Finally, from the standpoint of *senescence* of flight ability, the steady diminution in the capacity of male house flies to sustain the original high levels of flight intensity (WBF) for extended periods of time confirms previously obtained quantitative, time-related data for wing loss as such, and decline in ATP-ase, alpha-glycerophosphate dehydrogenase and acid phosphatase activity, both in the sarcosomes and in the extrasarcosomal elements of the flight muscle (Rockstein, 1956; Rockstein and Brandt, 1963).

SUMMARY

1. The age-related changes in wing beat frequency and duration of flight were studied in senescent male and female NAIDM house flies.
2. The average wing beat frequency increases to a maximum by the fifth day in female and by the fourth day in male house flies.
3. Duration of flight shows a steady, day-to-day decline with age. In females, this falls from 500 minutes for one-day-old to 110 minutes for 22-day-old flies. For males, this drop is more striking, with duration of flight falling from 420 minutes in one-day-old to 63 minutes for nine-day-old males.
4. These findings correspond to and confirm quantitatively previously reported data for wing loss and similar age-related changes in enzyme and coenzyme content in the flight muscle of senescent house flies.

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ARTIFICIAL CULTURE OF MARINE SEA WEEDS IN RECIRCULATION AQUARIUM SYSTEMS

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Purely artificial sources of sea water at present are still totally inadequate to facilitate normal and prolonged growth of any of the more exacting marine sea weeds (Provasoli, 1963, p. 9). Enriched sea water techniques, however, have provided a suitable means to establish speciation and life-cycle determinations of several highly organized forms. In 1934 Föyn successfully cultured *Ulva lactuca* through a complete life-cycle in enriched media, using "Erdschreiber" sea water with N, P, and soil extract. Subsequent studies on artificial culture of *Ulva* and related genera have developed through empirical refinement of this basic technique.

Provasoli (1963, p. 11) was able to obtain nearly natural morphological development, *i.e.*, a flat blade-like thallus 2-3 cm. long, in sporelings of *Ulva lactuca* only with initial samples of enriched sea water. Subsequent samples enriched in the same manner yielded varying results. The inability to provide for sustained growth and development in a reproducible, synthetic medium has thus greatly restricted our understanding of salient ecological and physiological processes involved in growing marine sea weeds. Specific disadvantages of older enrichment methods are obvious, and they include: significant variability in micro-element composition of different sea water samples; reliance upon heat sterilization to provide contaminant-free media, often resulting in precipitation of essential additives; and variability in soil extract samples utilized.

It is evident that current methods for simulation of natural conditions are far from adequate. Information available provides exhaustive description of the uses of trace elements, growth regulators, and vitamin sources, but specifically fails to provide fresh insight into the complex of physical, chemical, and biological factors and the way in which they alter the bathing medium. In many respects oceans are relatively constant biological environments, for example, with respect to dissolved gases, salinity, pH, temperature, and illumination (Provasoli *et al.*, 1957). Wider variations are evident in littoral or estuarine waters, but again, transitions are probably gradual and seasonal under most natural conditions. In a laboratory situation these properties can be controlled readily. Biotic interaction and micro-element composition present additional complications, but in principle at least can be brought under laboratory control. For the work reported here, a closed-system approach was implemented to facilitate systematic study of essential parameters.

MATERIALS AND METHODS

Marine sea weeds utilized in present investigations were gathered from Monterey Bay, Moss Beach, Point Reyes, and San Francisco Bay. Most species col-

lected were accurately identified in the field. Verification of closely related forms was achieved through standard histological technique. A fixing fluid consisting of 1 g. chromic acid, 1 ml. propionic acid in 90 cc. sea water proved to be satisfactory for most marine algae. Delafield's or Harris' hematoxylin was a suitable stain for most preparations; an appropriate counterstain of erythrosin B, orange G (0.8%) or fast-green (Johansen, 1951, pp. 359, 361) was also used.

Transplants, prior to placement into aquaria, were rinsed in fresh sea water and pesticide, using Lindane (hexachloro-cyclohexane), 5 ppm. for 15 minutes,

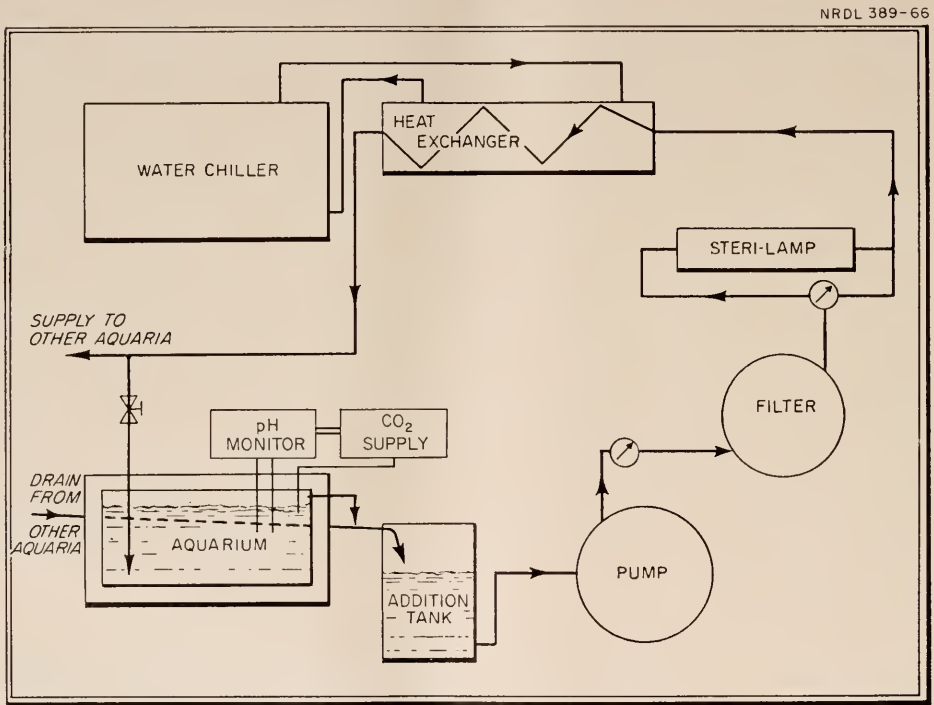


FIGURE 1. Flow diagram for recirculation aquarium system: temperature $16 \pm 1^\circ \text{C}$.; salinity $25 \pm 3\text{‰}$; total filtration from 4 to 10μ ; pH 7.9 ± 0.3 ; recirculation 190 L./min.; sea water replacement 4% per day. Details described in text.

to kill resident herbivorous gastropods and polychaetes. Polarographic measurements at the surface of *Ulva* after 24 hours exposure indicate that Lindane at concentrations of 650 ppm. did not appreciably inhibit oxygen evolution (report in preparation).

Of eight requirements to obtain optimal growth, five were adjusted empirically: (1) micronutrient enrichment, (2) CO_2 as a carbon source, (3) salinity and pH stabilization, (4) light source of suitable intensity and spectral quality, and (5) agitation and aeration. Additional requirements were necessitated due to the recirculation of sea water within the system: (6) nonmetallic construction, (7) filtration and sterilization, and (8) sea water replenishment. A schematic diagram of

the closed-recirculating aquarium system is shown in Figure 1; its components will be described separately below:

Fresh, sand-filtered sea water from off-shore was provided by Steinhart Aquarium, California Academy of Sciences, and this was enriched with phosphate, nitrate, and EDTA and other micronutrients. A mixture like that of Haxo and Sweeney (Provasoli *et al.*, 1957) provided a medium favorable to growth of *Ulva lobata* and related species, except that thiamin, biotin, and B₁₂ were substituted for soil extract (Table I). Tris (hydroxymethyl) aminomethane buffer, pH range 7.5–8.5, also was added at 0.3 part per thousand. The salinity of fresh sea water with its micronutrients then was adjusted to 25 ± 3.0 parts per thousand. Daily replacement was maintained at the rate of 4% of the total volume of the aquarium system.

TABLE I
Sea water enrichment mixture

KNO ₃	20.0 mg.
K ₂ HPO ₄	3.5 mg.
FeCl ₃	0.097 mg.
MnCl ₂	0.0075 mg.
Glycerophosphate di-sodium pentahydrate	1.0 mg.
EDTA	1.0 mg.
B ₁₂	1.0 μ g.
Thiamin HCl	0.2 mg.
Biotin	1.0 μ g.
Tris (hydroxymethyl) aminomethane	30.0 mg.
Fresh off-shore sea water	75.0 ml.
Distilled water	25.0 ml.

Vitamins, organic phosphate, and Tris buffer not included in Haxo and Sweeney formulation (1955).

The recirculating aquarium system was constructed of fiberglass, polyvinyl chloride (PVC), hard rubber, plastic, and glass, in order to reduce to a negligible degree contamination by undesirable metallic ions.

Water entering the closed system passed initially through a 20 μ mesh pre-filter within the addition tank. Water leaving the addition tank passed through the pump (Duriron Company, Inc.) into a filter column containing a non-impregnated cellulose cartridge. The cartridge was designed to remove particles within a 4 to 10 μ range (Hilliard-Hilco Corp.). Efficient filtration was achieved at flow rates in excess of 190 liters per minute, and at pressures not exceeding 60 psi. Temperature control at $16 \pm 1^\circ$ C. was maintained by a two-stage system. The initial level was a heat exchanger in which 10–20% ethylene glycol in water was cooled with freon refrigerant by a 5-ton capacity compressor (Dunham-Bush, Inc.). The ethylene glycol solution in turn exchanged heat through an impervious graphite shell and tube heat exchanger, the latter of which circulated aquarium water (National Carbon Co.).

Alkalinity was monitored continually, and it was automatically stabilized at pH 7.9 ± 0.3 by continuous metering of CO₂ gas. For this purpose, standard pH electrodes were placed just below surface water. A pH meter (Beckman, Model

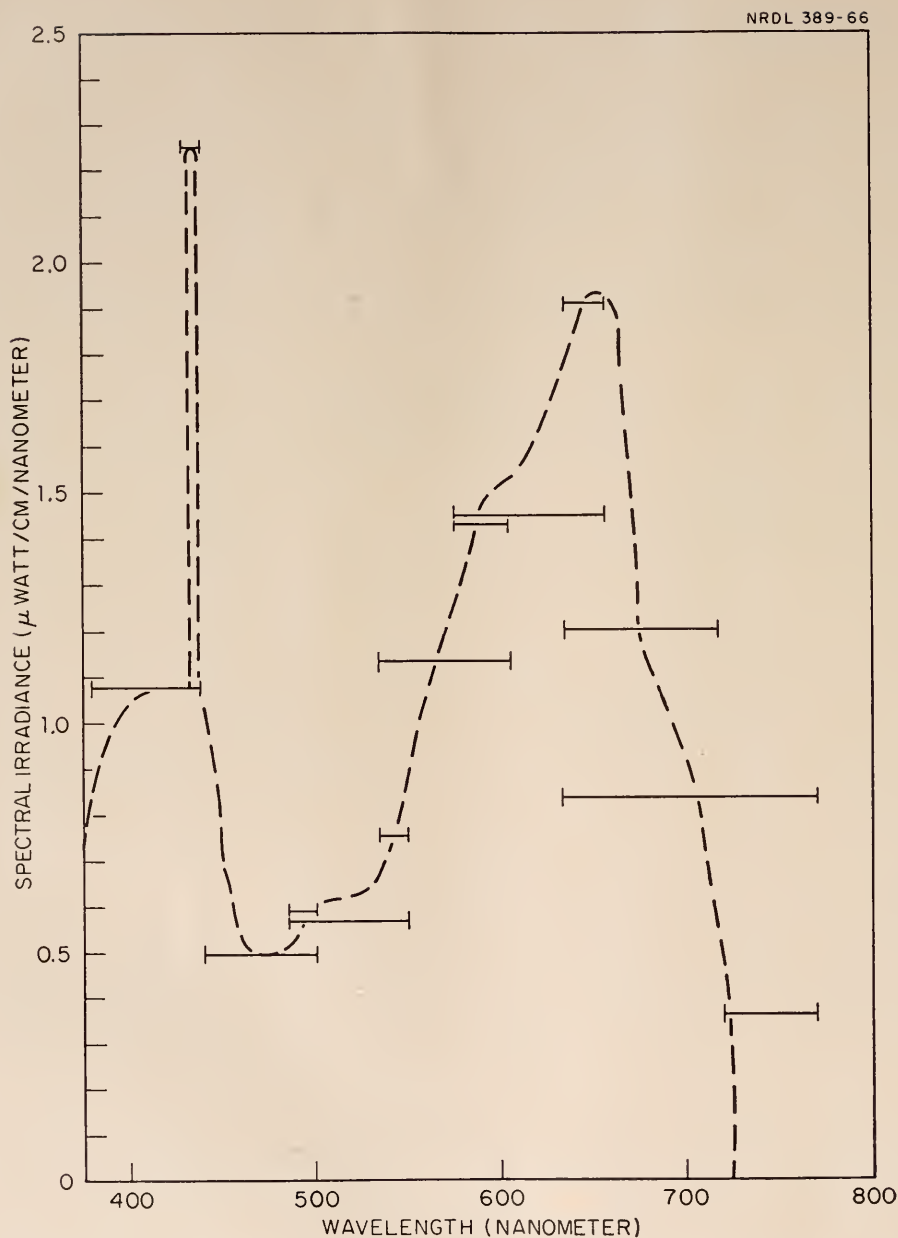


FIGURE 2. Spectral energy distribution (S.E.D.) at water surface (dotted line); energy detected through wide and narrow band-pass interference filters (horizontal bars). S.E.D. curve was interpolated, amounting to approximately 3.7 kilolux as the integrated area from 375 to 725 nanometers. For combined source, using 40 watts Gro-lux and 40 watts warm white (fluorescent). Details in text.

H-2) was modified by replacing the indicating meter with a meter relay switch. This switch activated a solenoid valve on the CO₂ cylinder when pH increased. Gaseous CO₂ entering the system in this manner not only enhanced pH stabilization but also provided an additional carbon source for photosynthesis.

Contaminating bacteria, protozoa, and diatoms were controlled through use of an ultraviolet sterilization source. This unit was constructed entirely of PVC and quartz glass tubing, discharging radiations in the 2537 Å range (Aqua-Fine Corp.). The unit efficiently killed biotic contaminants after 24 hours of recirculation, at flow rates in excess of 190 liters per minute. Elimination of biotic contaminants was confirmed bacteriologically.

Overhead lighting was provided for each aquarium. Two 40-watt, fluorescent lamps, 1 Gro-lux (Sylvania), and 1 Warm-white (General Electric), were placed in a standard white, painted reflector at the spacing recommended by the manufacturer (Mpelkas, 1964a). This, when located 50 cm. above aquarium top, yielded an irradiance almost entirely in the visible region, amounting to 3.7 kilolux at the water surface (Fig. 2). Energy emission of the Gro-lux lamps follows closely the absorption spectrum of chlorophyll pigments with peak energy output in the 440–460 and 660–680 nanometer range (Mpelkas, 1964b). Peak emission of the Warm-white lamp occurs within the mid-region of the action spectrum, 490–590 nanometers (Mpelkas, 1964b) fitting the absorption spectra of accessory pigments. Spectral energy distribution for the combined sources at 50 cm. is reported in Figure 2. This was determined using narrow band pass interference filters (Set 60; Optics Technology, Inc.) in line with an optical power meter (Model 610; Optics Technology, Inc.). Response characteristics of the meter-filter system were standardized against a known source for spectral irradiance. The photoperiod was maintained automatically, and was varied according to conditions to be described at a later point. Culture tanks were installed in a windowless room, devoid of natural sunlight.

RESULTS

Taxonomy and natural history of Ulva lobata

Thalli, like those described by Setchell and Gardner (1920) and Smith (1944) were found along the California Coast; they attained moderate size, nearly 50 cm. tall, 20–30 cm. broad, and were usually rich green in color. The plants observed were saxicolous and occasionally epiphytic, and they were found in the mid-littoral zone between 2.0- and – 2.0-ft. tide levels. Blades were membranaceous, broadly expanded, deeply divided, and slightly ruffled at margins. The thalli gradually narrowed to a stipe-like holdfast. Holdfast structures were perennial; blade portions, annual. This species differed from the closely related *Ulva expansa*, primarily in extent of division and size of blade. The latter was not deeply divided and was observed to attain a length in excess of 150 cm. Like those of all species of the genera, the blade of *Ulva lobata* was distromatic. It varied from 40 μ thickness at the margin to 90 μ in the more central portion. Cells as examined microscopically are shown in Figure 3.

The literature indicates that: reproduction follows an alternation of identical asexual and sexual generations; each fertile cell of the diploid sporophyte is capable

of producing 8 or 16 quadri-flagellate zoospores; meiosis occurs during the first and second divisions, the zoospores developing into haploid gametophytes; and that the gametophyte generation is heterothallic and anisogamous (Smith, 1944). Gametes released by mature plants were measured in the present study by stage

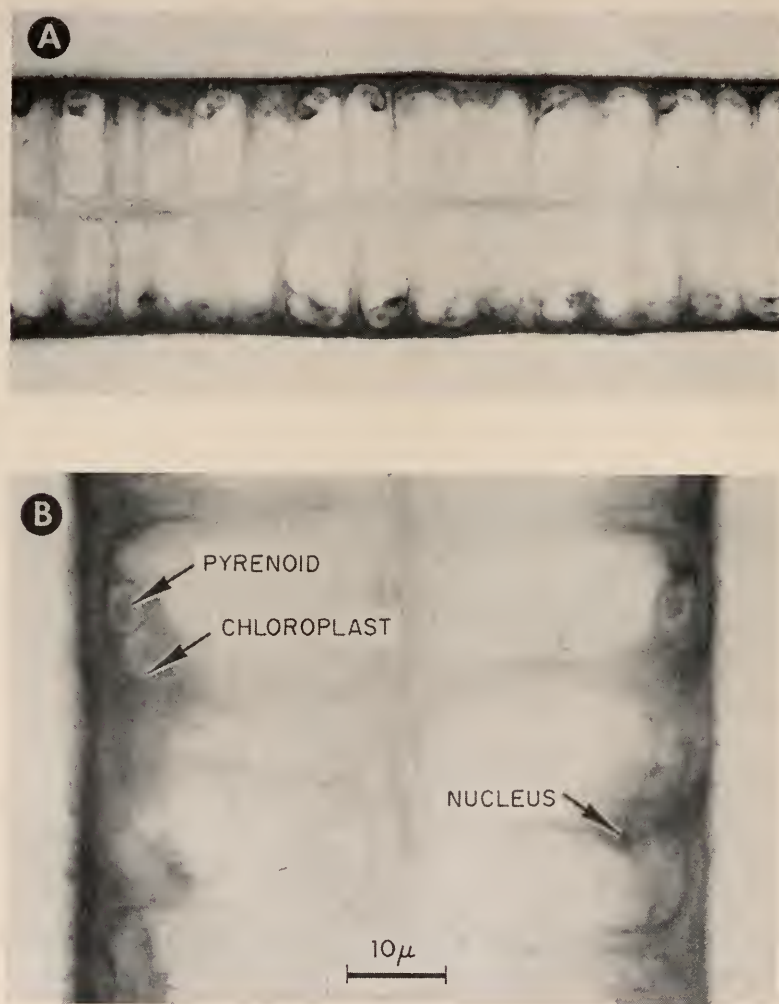


FIGURE 3. *Ulva lobata*, cross-section, H&E stain. Cells appear tightly packed with walls of adjacent cells confluent to form a rigid matrix. Note distromatic organization. (A) $\times 760$, (B) $\times 1900$.

micrometer, verifying reported descriptions of anisogamy. Male gametes here measured $2.0\text{--}3.5 \times 5.5\text{--}7.0 \mu$ in size; female gametes measured $2.5\text{--}4.0 \times 6.0\text{--}8.0 \mu$. Each was ovate or pear-shaped, showing a conspicuous eyespot and chloroplast, and each was bi-flagellate.

Gametes and zoospores were observed to be discharged through a small pore on the external border of marginal cells. The exit pore was not observed until the onset of sporulation. Instantaneous with release, gametes or zoospores collected into clumps, containing often as many as 50–100 cells. This clumping was somewhat larger than that reported by others (Smith, 1947). Gametic union occurred during clumping, pairs fusing side-by-side or anteriorly end-on-end. Clumps disintegrated within 2 to 3 minutes, and the quadri-flagellate zygotes remained motile for several hours. Zoospores were observed to remain motile longer, *e.g.*, 4 to 5 hours.

Available literature (Smith, 1947) shows that fruiting or sporulation of both gametophyte and sporophyte generations occurs at predictable 28-day intervals, but only during the spring tides of the lunar month, as observed during summer months. Gametophytic plants sporulate early during the series of spring tides, while sporophytic plants liberate zoospores late during the series. Thalli of both generations are usually found closely associated and in approximately equal numbers. Gametes have been reported to germinate parthenogenetically (Smith, 1947; Moewus, 1938; and Yamada and Saito, 1938).

Aquarium development and differentiation of Ulva lobata

Fertile thalli of *Ulva lobata* were placed into plastic bags containing fresh sea water and transported to the laboratory in ice chests maintained at 10–15° C. Mature plants transported in this manner often discharged swarmers (gametes or zoospores) within a few hours, as they also did when placed into fresh sea water of normal seasonal temperature. The slightest change in environmental conditions often stimulated sporulation, for example decreasing temperature, desiccation or stimulation by intense light. Contents of bags showing spore liberation were mixed and gently agitated for one hour to insure fertilization. The resulting zygospores were poured into a 225-liter culture tank and water turnover was reduced to allow their implantation on the aquarium bottom.

Infertile thalli usually developed to maturity within a few days after placement into laboratory aquaria. Occasionally blades would sporulate spontaneously, usually 2–3 weeks after transplant. Even as late as December or January infertile thalli were seen to develop mature reproductive cells and could be stimulated to release swarmers.

Development of sporelings was followed on a daily basis and compared to existing accounts of similar species. Photomicrographs were prepared to record the significant stages of differentiation not previously described for *Ulva lobata* (Fig. 4). Development of the sporophyte generation will be described below.

One to two days after fertilization and implantation, zygotes became more spherical, approximately 6–8 μ in size. Two eye-spots and a single pyrenoid were still recognizable (Fig. 4A, B). In from 2 to 4 days, zygotes increased their size to 8–10 μ and showed a thin, clear membrane which gradually thickened as the onset of germination approached. Germination occurred in from 6 to 7 days and the eye-spots at this stage were no longer visible. At 9 to 10 days, the sporeling appeared as a slender filament of 8–10 cells, 40–45 μ in length, since division only occurred along the transverse axis (Fig. 4C). At this time, the basal cell sometimes elongated to form a primary rhizoid. At 14 days, and at nearly 100 μ in

length, the first cellular divisions along the longitudinal plane of the filament occurred (Fig. 4D); secondary rhizoids were then observed to develop. Through continued longitudinal divisions, at 40 days, the sporeling represented the flattened membranaceous structure of the mature thallus. At 60 days, the young plant was 4.5–5.0 cm. in length and 1.5–2.0 cm in breadth (Fig. 4E). Although the young sporophytes were of sufficient size and maturity to liberate zoospores, subsequent development of the gametophyte generation was not followed. In these

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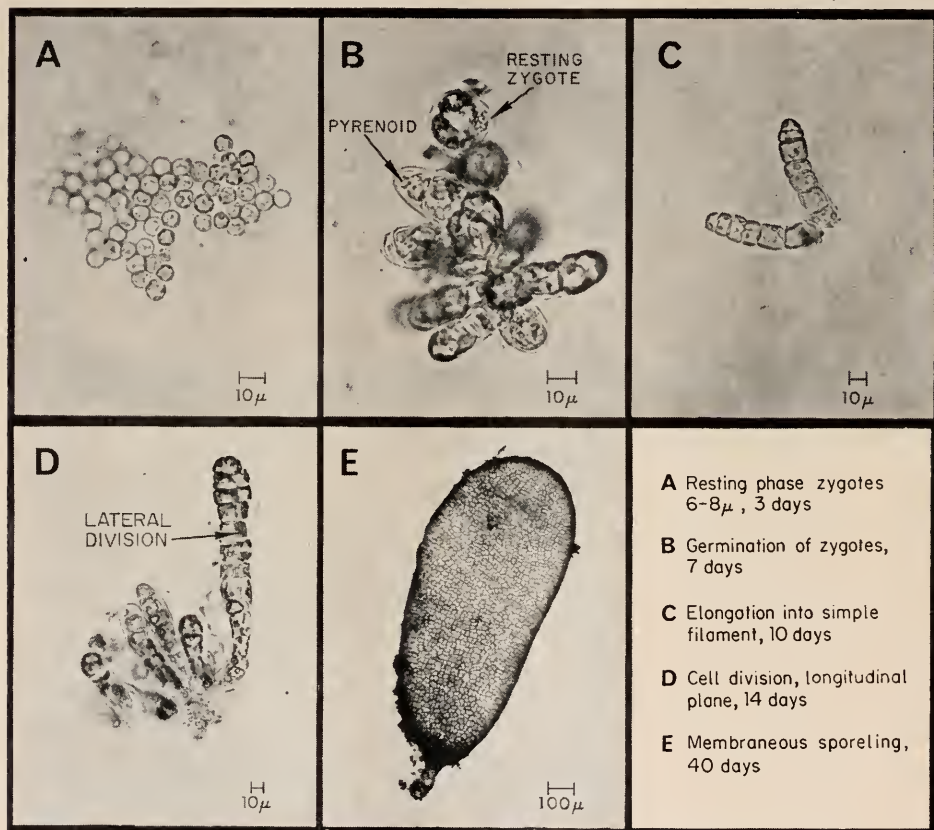


FIGURE 4. Photomicrographs showing development and differentiation of *Ulva lobata* sporelings in recirculation aquarium system.

studies it was not possible to distinguish morphological differences in the development of *Ulva lobata*, when compared to the closely related species, *Ulva (lactuca)* (Miyake and Kunieda, 1931) or *Ulva pertusa* (Yamada and Saito, 1938). Normal elongation and subsequent differentiation were clearly demonstrated in Figure 4. Deformities such as the acute twisting and bulging of *Ulva lactuca* filaments, described by Rea (1964), were not evident.

Longitudinal division of filaments of *Ulva lobata* occurred in approximately 10–14 days here, whereas in a study of *Ulva pertusa*, it occurred no earlier than

30 days after germination (Yamada and Saito, 1938). Also, in the present study sporelings were nearly ten-fold larger at 45 days, compared to those of *Ulva pertusa* as measured at 60 days. These differences probably do not indicate different species characteristics, but rather attest to the adequacy of the present culture conditions. It seems likely that earlier accounts of sporeling development were hampered by less adequate growth conditions.

Laboratory-maintained Ulva transplants

The genus *Ulva* demonstrates a marked seasonal periodicity with respect to both vegetative and reproductive growth, and this is mediated undoubtedly through both environmental and less understood internal factors. Among the former, duration of photoperiod and water temperature are of particular interest.

In general, vegetative growth in *Ulva* is observed to begin slowly in early Spring with the known lengthening of photoperiod and rise in water temperature. Maximum attainment of both vegetative and reproductive processes is reached by mid-summer. The growth response gradually diminishes by late summer, the thallus dying back to where only the holdfast and a small portion of the leafy blade survive the winter months. This attrition seems to be determined by changes in duration of photoperiod and environmental temperature. It affects both gametophyte and sporophyte generations alike.

Care was exercised during collection of specimens not to damage or disrupt holdfast structures. Usually small plants, less than 20 cm. tall and firmly anchored to small rocks or pebbles, were selected. Thalli in which holdfasts were carefully cleaved from attachment occasionally would continue differentiation of this structure and subsequently reattach on aquarium bottoms. Usually samples of *Ulva lobata*, *Ulva lactuca*, and *Ulva linza*, with holdfasts intact, transplanted better; however, this was not true of *Ulva expansa*. In the latter species, free-living thalli frequently break away from the holdfast and continue to grow, if free-floating in more quiet backwaters (Smith, 1944).

Seasonal rhythm was preserved during laboratory culture and was paramount in determining transplant longevity (Table II). If viable plants were removed for transplant early during the growth season, that is, during May or June, they were usually adequately maintained for three months or more, comparable to the natural seasonal growth. Thalli introduced to laboratory culture late during the growth season exhibited a less extensive vegetative development or none at all. Exposure to those environmental conditions correlated with maximal vegetative and reproductive development did not alter the onset of senescence; *e.g.*, water temperature (surface) 16–18° C. and photoperiod 13–15 hours, which are mean summer conditions in Monterey Bay. Long-day illumination, 13–15 hours, under present methods of culture favored longevity of *Ulva* transplants removed from the sea during the months of May, June and July (Table II). Thalli removed during late summer, August and September, were maintained only if the photoperiod was decreased to 10–12 hours. Exposure to photoperiods above 16 hours or below 10 hours caused a gradual degeneration of tissues in 7–10 days. Exposure to continuous light produces a rapid shriveling and hardening of the thallus, evident in 48 hours. Microscopic examination of tissues illuminated continually for 96 hours revealed a marked dissolution of mucilage material associated with the free

TABLE II

*Effect of photoperiod and other lighting conditions on longevity; summarized data for Ulva transplants maintained in recirculation aquaria**

Species	Transplants or sporel. (N)	Natural photoper. (hr.)	Artificial photoper. (hr.)	Source of illum.	Irrad. level (kilolux)	Longevity‡
May-Ju. collections:						
<i>U. lobata</i>	12	14.0	13-15	GL+WW	3.7	3 mo.
<i>U. lobata</i>	12	14.5	16-18	GL+WW	3.7	1 wk.
<i>U. expansa</i>	12	14.5	16-18	GL+WW	3.7	1 wk.
<i>U. lobata</i>	4	14.5	continuous	GL+WW	3.7	48 hr.
<i>U. expansa</i>	4	14.5	continuous	GL+WW	3.7	48 hr.
<i>U. lobata</i>	12	14.5	13-15	GL+WW	7.4	1-2 wk.
Oct. collections:						
<i>U. lobata</i>	12	11.5	10-12	GL+WW	3.7	2 mo.
<i>U. expansa</i>	12	11.5	10-12	GL+WW	3.7	2 mo.
Sporelings, <i>U. lob.</i>	300 ^Δ	11.5	13-15	GL+WW	3.7	3 mo.
Sporelings, <i>U. lob.</i>	300 ^Δ	11.5	13-15	GL+WW	7.4	3 mo.
<i>U. lobata</i>	12	11.5	13-15	GL+WW	3.7	1-2 wk.
<i>U. expansa</i>	12	11.5	13-15	GL+WW	3.7	
<i>U. lobata</i>	12	11.5	8	GL+WW	3.7	1 wk.
<i>U. expansa</i>	12	11.5	8	GL+WW	3.7	1 wk.
<i>U. lobata</i>	24	11.5	10-12	DL+DL	3.7	1-2 wk.
<i>U. expansa</i>	24	11.5	10-12	DL+DL	3.7	1-2 wk.
<i>U. lobata</i>	24	11.5	10-12	CW+CW	3.7	1-2 wk.
<i>U. expansa</i>	24	11.5	10-12	CW+CW	3.7	1-2 wk.
Dec.-Jan. collections:						
<i>U. lobata</i>	24	10.0	10-12	GL+WW	3.7	2 mo.
<i>U. expansa</i>	24	10.0	10-12	GL+WW	3.7	2 mo.

* Nutrient factors, salinity, pH and other conditions are constant; see text.

† Forty-watt fluorescent tubes: GL = Gro-Lux; WW = Warm-white; DL = Daylight; CW = Cool white.

‡ Bleaching and autolysis first evidenced; for continuous illum., dissolution of mucilaginous envelope and shriveling of thallus were evidenced at 48 hours.

^Δ Number estimated from area and number in microscopic field.

cell border surfaces of the blade. Comparable tissue breakdown occurred if light intensities exceeded 3.7 kilolux, although sporelings of *Ulva lobata* tolerated intensities at a level of 7.4 kilolux, as well as a longer photoperiod.

Unbuffered aquarium waters showed the well known rapid decrease in hydrogen ion concentration when transplants were illuminated (Shelbourne, 1964, p. 29). During dark periods pH values fell, but not to initial levels: thus, total alkalinity gradually increased within 5-7 days to pH values in excess of 9.0. Sustained

alkalinity of this magnitude usually resulted in a progressive degeneration of transplanted thalli. Comparable effects resulted if pH values were maintained below 7.5.

DISCUSSION

Possible seasonal change in the water occurring along the mid-California coast did not affect the success of present cultures. Sterilization of media by autoclaving was not necessary to avoid gross biotic infection, as effective control was achieved by ultraviolet irradiation. This also permitted use of a wider salinity range, as well as greater concentrations of both inorganic and organic constituents precipitable by heat sterilization. Vitamins, chelators, and other organics could be added to media precisely with results comparable to those using soil extracts. While the B vitamins, thiamin, B₁₂, and biotin, have been shown to stimulate growth of many unicellular algae including diatoms their role is less understood for marine sea weeds. Of the Rhodophyceae, *Goniotrichum*, *Nemalion*, *Antithamnion*, and *Bangia*, all are found to utilize one or more of the B vitamins in laboratory culture (Provasoli, 1963, p. 13). Kylin (1942) reported enhanced growth of both *Ulva* and *Enteromorpha* with thiamin at an optimal concentration of 10 mg./liter. None of the Phaeophyceae thus far studied are known to require these additives, but since insufficient information is available, it seemed inadvisable to omit these constituents.

Tris (hydroxymethyl) aminomethane at 0.03% concentration in the system buffers adequately between pH 7.5 and 8.5. Thus, pH levels in the aquaria were stabilized at 7.9 ± 0.3 , with gaseous CO₂ (1–10%) bubbled through the medium intermittently. Provasoli (1957) indicated that Tris (0.1%) was not inhibitory to the most sensitive of marine algae, and in the present work, comparable concentrations were not inhibitory to either transplants or developing sporelings of *Ulva*. EDTA at a final concentration of 3×10^{-5} M is sufficient to bind the trace elements in sea water (Johnston, 1964). EDTA has the added advantage of being metabolically inactive for most organisms, and it evidently does not promote growth of contaminating biota within non-sterile media (Hutner *et al.*, 1950).

In maintaining proper alkalinity levels, ultraviolet sterilization and microfiltration supplanted chemical buffering by eliminating bacterial and other organic growth which would otherwise acidify the medium. Many sea weeds are found to be tolerant of wide fluctuations in pH, due primarily to tidal influence. Blinks (1963) demonstrated that photosynthetic rate decreased by 50% only, when fronds of *Ulva* and *Enteromorpha* were maintained for about 6 hours at pH 9.8 or above. Kylin (1927) indicated that certain intertidal sea weeds survived for 1–3 days within the pH range of 6.8 to 9.6. From the present studies, it would appear that *Ulva* and related genera are more closely restricted in pH requirements than might first be suspected, and that even high ranges as encountered in isolated tide pools would be inhibitory if sustained longer than normal tidal influence allows.

Typically *Ulva lobata* and related species are long-day, short-night plants. At an irradiance of 3.7 kilolux, illumination for 13–15 hours and uninterrupted darkness for 9–11 hours favor longevity of transplants. In nature, both vegetative and reproductive development reach maturity during similar long-day seasons. Also it is noted that lunar periodicity governs the release of gametes and zoospores, undoubtedly correlated to the 24-hour light-dark cycle (Smith, 1944).

From our field observations, water temperature seemed to have a greater effect on the rapid maturation of gametes and zoospores than duration of photoperiod. Although approaching maximum vegetative growth in late spring, fertile thalli were not evident until water temperatures reached summer levels, 16–18° C. It followed that incidence of fertile plants decreased abruptly during early September as water temperatures within the vicinity of collection sites fell below 16° C. That the response was a consequence of temperature change and not a consequence of the shortened photoperiod was shown by the following observation. Placing infertile plants collected as late as December or January into aquaria maintained at 16–18° C., with an artificial photoperiod of 9–10 hours, did induce subsequent maturation and sporulation (data not reported).

The influence of temperature on reproductive growth of many plants is closely interrelated with photoperiodism. Depending upon the species of plant and other conditions, temperature may enhance or oppose the effect of photoperiod on reproductive maturity (Meyer and Anderson, 1952, p. 681). Temperature changes may initiate synthesis or breakdown of hormonal compounds, rates of translocation, and relative effectiveness of specific morphogenic change (Meyer and Anderson, 1952, p. 681). Aquatic plants such as *Ulva* perhaps have biochemical similarity to terrestrial plants, since comparable phyto-hormone responses are present (Provasoli, 1957, 1958), and since metabolic effects can be triggered by photoperiod and temperature changes like those known to depend on phyto-hormone systems.

Scant information is available concerning either the intensity levels or the spectral quality essential to artificial maintenance of marine sea weeds. An intensity range of 1.1 to 5.4 kilolux has been reported for Rhodophyceae (Provasoli, 1963, p. 10; Iwasaki, 1961). Föyn (1934, 1960), however, with *Ulva* did not report intensity ranges. Other investigators have simply exposed cultures to natural sunlight. Investigations by Dellow and Cassie (1955) on the littoral zonation in caves demonstrated the adaptability of such forms as *Cladofora* to grow normally at low intensity levels, in the range of 0.005 to 0.250 kilolux. In similar experiments, the intertidal alga, *Ulva lobata*, could survive 5 hours exposure to direct sunlight (104 kilolux) (Biebl, 1952). However, in the natural environment, constantly changing intensities, attenuation of red, and scattering of blue wave-lengths, are all complicating factors.

While the energy distribution of the Sylvania Gro-lux fluorescent lamp approximated a photosynthetic action spectrum (Mpelkas, 1964b), the combined sources here used provided a better spectral balance for growth and differentiation. Haxo and Blinks (1950), in comparing light absorption and photosynthetic action spectra in *Ulva* and *Monostroma*, demonstrated that higher rates occurred in spectral bands corresponding to absorption by chlorophyll A, at 435 and 675 nanometers. In our studies, when *Ulva* transplants were illuminated at comparable intensities but with Gro-lux lamps alone or with Cool-white or Daylight lamps, the transplants gradually bleached and decayed. These adverse changes presumably were due to inadequate energy emission in the red spectral regions when the sources indicated were used individually (Mpelkas, 1964; Haxo and Blinks, 1950). For the combined light sources finally adopted, abrupt drop in energy distribution below 400 and above 725 nanometers (Mpelkas, 1964b) lessens concern about possibly unfavorable emissions, *e.g.*, mercury lines, ultraviolet and infrared wave-lengths.

With little modification, the basic system has facilitated cultures of other marine forms, *e.g.*, sea weeds such as *Porphyra perforata*, *Polyneura latissima* and *Schizymenia pacifica*, and diatoms such as *Ditylum brightwellii*, *Nitzschia angularis* and *Navicula* (sps. und.). It has also permitted successful culture of such animal forms as protozoa, annelids, molluscs and crustaceans.

The authors are grateful for the kind assistance of Mr. Clay P. Butler of this Laboratory, who standardized their tungsten source against NBS calibrated lamp QL-50 (tungsten).

SUMMARY

Ulva as either sporeling or transplant could be cultured for periods of three months in closed recirculating aquarium systems. Early development of *Ulva lobata* sporelings proceeded normally and rapidly under conditions imposed and compared to closely related species, *i.e.*, *Ulva pertusa* and *Ulva lactuca*. A modified Haxo-Sweeney enrichment was used, substituting B vitamins and organic phosphate for soil extract. Continuous flow ultraviolet sterilization and microfiltration were provided. The pH was maintained automatically at 7.9 ± 0.3 , using Tris buffer and gaseous CO_2 . Improved fluorescent illumination for 13–15 hours favored culture of sporelings and summer transplants. Irradiance was confined to the spectrum lying between approximately 380–725 $\text{m}\mu$, and amounted to 3.7 kilolux. From field observations, photoperiod appeared closely correlated to initiation of vegetative growth during early spring. Water temperature seemed to have a greater effect on the rapid maturation of gametes and zoospores.

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THE MORPHOLOGY AND LIFE-HISTORY OF NOTOCOTYLUS
ATLANTICUS N. SP., A DIGENETIC TREMATODE OF EIDER
DUCKS, SOMATERIA MOLLISSIMA, AND THE DESIG-
NATION, NOTOCOTYLUS DUBOISI NOM. NOV.,
FOR NOTOCOTYLUS IMBRICATUS (LOOSS,
1893) SZIDAT, 1935¹

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The genus *Notocotylus* was erected by Diesing (1839) with *Notocotylus triserialis* Diesing, 1839, from wild and domestic European ducks, as type species. Diesing confused dorsal and ventral aspects and, as the generic name implies, regarded the characteristic pits as dorsal. The genus is worldwide in distribution and contains species from various birds and mammals. On the presumption by Creplin (1846) that *N. triserialis* is identical with *Monostomum attenuatum* Rudolphi, 1809, from *Scolopax gallinago*, Kossack (1911) designated *Notocotylus attenuatus* (Rudolphi, 1809) as type of the genus and this determination has been accepted by most subsequent authors. However, Dubois (1951) questioned the identity of the two species and presented strong evidence to support his contention that *N. attenuatus* is not identifiable and that it should be considered a "species inquirende." Accordingly, he restored *N. triserialis* as type of the genus.

The generic concept of *Notocotylus* is vague, uncertain, and the diagnostic criteria are disputed. Many species, named and described as new, have been suppressed as synonyms and the number of valid species is problematical. Yamaguti (1958) listed 29 species from birds and five species from mammals, including *Notocotylus urbanensis* (Cort, 1914) Harrah, 1922, which has been reported from experimental infections of both birds and mammals (Luttermoser, 1935; Herber, 1939, 1942; Acholonu, 1964). Dubois (1951) recognized 19 species and Odening (1964) included 26 species in a differential key. Certain species have been designated as types of new genera, but these proposals have not been adopted. As synonyms of *Notocotylus*, Yamaguti (1958) listed *Hindia* Lal, 1935; *Naviformia* Lal, 1935; *Kossackia* Szidat, 1936; and *Hindolana* Strand, 1942. Baer and Joyeux (1961) suppressed *Hippocrepsis* Travassos, 1922; *Hindolana* Strand, 1942 (= *Hindia* Lal nec Duncan); *Quinqueserialis* Skwartzow, 1933 (= *Barkeria* Szidat, 1936) and *Uniserialis* Burton, 1958 as synonyms of *Notocotylus*. Revisions of the genus have been made by Kossack (1911), Harrah (1922), Szidat and Szidat (1933), Harwood (1939), Dubois (1951), and Odening (1964); the last two contain extensive bibliographies.

Several life-history studies have been reported but the data are confusing, since the specific identity of the adults is uncertain and the correlation of larval

¹ Investigation supported by Grant NSF-GB-3606, continuation of G-23561.

and adult stages with the type of snail intermediate host is obscure. Some of the snail hosts are pulmonates, some are prosobranchs; some are fresh-water and others are marine. Sewell (1922) erected the "Ephemera Group" of monostome cercariae, based on *Cercaria ephemera* Nitzsch, 1807 from *Planorbis corneus* Linn., taken near Halle, Germany. Marie Lebour (1907) described cercariae from the marine prosobranch snail, *Peringia ulvae*, as *Cercaria ephemera*. Sewell predicated that *C. ephemera* Lebour could not be identical with *C. ephemera* Nitzsch and stated (p. 40), "Although nothing is definitely known regarding the ultimate development of any of these monostome forms, several suggestions have been put forward by different authors." After the description of more than 50 species belonging to various monostome, amphistome and distome groups, he declared (p. 309), "The classification of larval trematodes cannot be based on any one morphological system or groups of characters, but must include a study of their development, as well as of the morphology of both parent and offspring." Similarity between the "Ephemera" cercariae and notocotylid trematodes had long been noted, but correlation between particular larvae and their adult stages remained uncertain. Joyeux (1922) reported that cercariae from *Planorbis rotundatus* Poirer, taken in a lake at the Bois de Verrières near Paris, developed in domestic ducklings to adult worms that were identified as *Notocotylus attenuatus* (Rudolphi, 1809). Eggs from these worms provided miracidia which infected laboratory-reared snails and afforded the first experimentally demonstrated life-cycle in the group. Harper (1929) reported that trioculate monostome cercariae from *Lymnaea peregra* and *Physa fontinalis* developed in ducklings to adults identified as *Notocotylus seineti* Fuhrmann, 1919. Mathias (1930) found that cercariae from *Lymnaea limosa* developed to adults in ducklings; these were determined as *N. attenuatus*. Further, he believed that the specimens of Joyeux, which were only about one-half as large, represented a related but different species. Lothar and Ursula Szidat (1933) reported that *Cercaria ephemera* Nitzsch, 1807 from *Planorbis corneus* developed into adults, which, in violation of the rules of nomenclature, they designated as a new species, *Notocotylus thienemanni*. A cercaria from *Lymnaea palustris* was described by them as a new species, *Cercaria vaga*. Ursula Szidat (1935) fed larvae from *Bithynia tentaculata*, identified as *Cercaria imbricata* Looss, 1893, to chicks and ducklings and recovered adults, strikingly like *N. attenuatus*, but designated erroneously as a new species, *Notocotylus imbricatus*. She remarked on the difficulty of distinguishing between these closely related cercariae on purely morphological grounds and (1936) insisted on the marked specificity of larval stages and snail hosts.

The first comprehensive study of the "Ephemera Group" was done by Miriam Rothschild when in 1932 she began an investigation of the larval trematodes of *Peringia ulvae* (Pennant, 1777) at the suggestion of Dr. Marie Lebour. Miss Rothschild (1935) published a note on the development and structure of the excretory system of *C. ephemera* Lebour, 1907 (*nec* Nitzsch, 1807). She (1936a) reported three species: *Cercaria* E, *Cercaria* F, and *Cercaria* G, in addition to *C. ephemera* and (1936b), discussed the development of normal pigmentation and its variations in *C. ephemera* and allied species. After studying infections in *P. ulvae* and *Hydrobia ventrosa* (Montagu, 1803), Rothschild (1938) arranged

the notocotylid cercariae in three groups, based on the form of the anterior transverse portion of the excretory vesicle. In the Monostomi group, the anterior transverse portion of the vesicle is a closed tubular circuit situated posterior to the cerebral ganglion and median eye-spot; it contained 12 described species, including two from *P. ulvae*. In the imbricata group, the anterior portion of the vesicle forms a loop, between the lateral eye-spots, which passes anterior to the cerebral ganglion and median ocellus; four described and one undescribed species from *Hydrobia ventrosa* were assigned to this group. In the yenchingensis group, there is an unpaired, finger-like diverticulum which extends anterior from the transverse portion of the vesicle; three described species were included in this group, together with two undescribed species from *P. ulvae* and another from *Littorina neritoides* (Linn.). Rothschild (1940) explained that the imbricata group was based on *Cercaria imbricata* Looss, 1896 and that *C. imbricata* Looss, 1893 from *Paludina impura* (= *Bithynia tentaculata*) in Germany is a *nomen nudum*, since the species was not described or figured, and that *C. imbricata* Looss, 1896 from *Melania tuberculata* Bourg in Egypt is distinct and different from *C. imbricata* in Germany. She stated that the cercaria from *B. tentaculata* which U. Szidat found to mature in ducks, and which she named *Notocotylus imbricatus*, was the one studied by Looss and his surmise that it is the larva of *N. attenuatus* was probably correct. Attempts to solve the life-cycles of these species, carried on for five years by Miss Rothschild, were only partially successful. She reported (1941) that cysts of the Monostomi and Yenchingensis groups were fed to laboratory-reared ducklings and two unidentified species of *Paramonostomum* were recovered from birds fed Yenchingensis metacercarial cysts.

In his revision of the genus *Notocotylus*, Dubois (1951) recognized two subgenera: *Hindia* Lal, 1935; parasites of Ralliformes, with 4–6 glands in the median row, cuticula unarmed or provided with minute spines, type, *Notocotylus gibbus* (Mehlis in Creplin, 1846), syn. *Kossackia* U. Szidat, 1936; and *Notocotylus* Dubois, 1951, parasites of Anseriformes, with 10–25 glands in the median row, cuticula armed, type, *N. triserialis* Diesing, 1839. According to him, the subgenus *Notocotylus* is composed of two distinct groups, in one of which the cercariae develop in pulmonate snails, in rediae which lack locomotor appendages (apophyses of the German authors); whereas the asexual generations of the other group develop in prosobranch snails in rediae that are provided with locomotor appendages. The first group was based on *N. triserialis* with *Notocotylus urbanensis* (Cort, 1914); *Notocotylus intestinalis* Tubanguui, 1932; *Notocotylus deflax* Harwood, 1939; and *Notocotylus stagnicolae* Herber, 1942, listed as synonyms. The second group was based on *Notocotylus imbricatus* (Looss, 1893) U. Szidat, 1935, with *Notocotylus lucknowensis* (Lal, 1935) and *Notocotylus anatis* Ku, 1937, listed as synonyms, while *Notocotylus magniozatus* Yamaguti, 1934 and *Notocotylus babai* Bhalero, 1935 (syn. *Notocotylus indicus* Lal, 1935) were distinguished only by relative extent of vitellaria and uterine loops. Referring to the subgenus *Notocotylus*, Dubois observed (p. 64), "Les deux groupes biologiques 'triserialis' et 'imbricatus' constituent, en réalité, deux espèces polymorphes au sein des Notocotylidés que U. Szidat (1935, p. 270) envisageait comme un ensemble de Trématodes phylogénétiquement jeune et susceptible, par conséquent, d'une différenciation progressive

à partir des 'races physiologiques' actuelles (1936, p. 234). L'examen du tableau comparatif des stades larvaires confirm cette manière de voir et ne lève aucune des difficultés rencontrée dans la systématique des vers adultes." Dubois examined the specimens of Joyeux (1922), developed in ducklings from cercariae of *Planorbis rotundatus*, and identified them as *Catatropis verrucosa* (Fröhlich, 1789).

Rothschild (1940) noted that *Cercaria imbricata* Looss, 1893, was from *Bithynia tentaculata* in Germany and that *C. imbricata* Looss, 1896, was from *Melania tuberculata* in Egypt. Apparently Dubois (1951) accepted the allocation by U. Szidat (1935) of *C. imbricata* Looss, 1893, to the genus *Notocotylus* as a validation of the specific name since he employed the combination, *Notocotylus imbricatus* (Looss, 1893). But *C. imbricata* Looss, 1893, from *Bithynia tentaculata* was named provisionally in a footnote, without description or figure and, accordingly, has no validity. The specific name, *imbricatus*, is valid only for the Egyptian species, and if the opinion of Dubois that *C. imbricata* is the larval stage of *Notocotylus aegyptiacus*, Odhner, 1905, is correct, the name of that species is *Notocotylus imbricatus* (Looss, 1896), not *N. aegyptiacus*, a name proposed by Odhner (1905) to replace *Monostomum verrucosum* Looss, 1896, nec Fröhlich, 1789. It is interesting to note that Lühe (1909) listed *C. imbricata* from *B. tentaculata*, but based the diagnosis on Looss' description of the African species. A genus can not have two species with identical names and the species whose larvae occur in *B. tentaculata* and which has been known as *N. imbricatus* (Looss, 1893) has no valid name. For it I propose the name, *Notocotylus duboisi*. According to Dubois (1929), it is distinct from the species from *B. tentaculata* described by him as *Cercaria Helvetica* I. Odhner (1963) compared the cercariae from *B. tentaculata* described as *C. imbricata* Looss, 1893, by Wesenberg-Lund (1934) and by Emmel (1943) with *Cercaria Helvetica* I, but was uncertain of their identity.

Recent studies have only partially clarified the situation. Herber (1955) studied the life-cycle of *N. urbanensis* (Cort). He noted errors in the accounts of Barker (1915) and Harrah (1922): stated that the species described by Barker in the text as *Catatropis fimbriata* and figured as *Catatropis filamentis* and the species described by Harrah as *Paramonostomum echinatum* were based on poorly preserved specimens of *N. urbanensis* and that both names should be suppressed as synonyms. According to Herber, the specimens described by Harrah (1922) as *N. urbanensis* are not conspecific with *Cercaria urbanensis* Cort, 1914, but comprise a heterogeneous collection of immature specimens and the only ones identified were referred to the genus *Quinqueresialis*.

Stunkard and Dunihue (1931) described specimens from an unidentified duck as *Notocotylus gibbus* (Mehlis in Creplin, 1846). Determination was based on Kossack's description of the original specimens. The species was found again and redescribed by L. and U. Szidat (1933). Harwood (1939) then assigned the specimens of Stunkard and Dunihue to *Notocotylus imbricatus* (Looss). Stunkard (1960) reported notocotylid cercariae from *Hydrobia minuta* taken near Boothbay Harbor, Maine, that encysted on the opercula of the snails and on empty shells of *Gemma gemma*. Encysted metacercariae were fed to laboratory-reared birds: chicks, eider ducks, herring gulls, and common terns, as well as white mice and hamsters. Infection was obtained only in eider ducks, *Somateria mollissima*, and

adult worms taken from the caeca proved identical with those described by Stunkard and Dunihue (1931). With the more complete description of *N. gibbus* by L. and U. Szidat, it was apparent that the specimens described by Stunkard and Dunihue differ from that species in host and geographical distribution, in size of worms, size and shape of gonads, and in the number and arrangement of ventral glands. The worms could not be assigned to *N. imbricatus* or any other known species and were described by Stunkard (1960) as a new species, *Notocotylus minutus*.

Determination of species in the genus *Notocotylus* is difficult. The worms are very similar (witness the keys by Harwood, 1939; Dubois, 1951; and Odening, 1964); specific differences in morphology are small, and the extent of individual variation is unknown. Host specificity of the adult stage is not definite, because the same metacercariae can mature in different host species. Acholonu (1964) reported the development of metacercariae of *N. urbanensis* and *N. stagnicola* (possibly identical forms) in ducklings, goslings, chicks, white rats and muskrats. Apparently the snail hosts are more specific, and U. Szidat (1935) predicated precise specificity in *C. ephemera*, *C. attenuatus*, and *C. imbricata*. But there is so much confusion in the identification of larvae that final conclusions are impossible. No less than five different cercariae have been described as larvae of *N. attenuatus* by Joyeux (1922), Mathias (1930), L. and U. Szidat (1933), Yamaguti (1938) and Wright and Bennett (1964), and the identity of *N. attenuatus* is precarious.

Other more recent studies on the life-history of notocotylid species include the report by Wu (1953) on the development of cercariae from *Stagnicola palustris* to adults of *N. stagnicola* in chicks; and by Erkina (1954) and Bychovskaja-Pavlovskaja (1962) on the development of *Notocotylus chionis* of the Soviet authors (Erkina, 1954 and Bychovskaja-Pavlovskaja, 1962) nec Baylis, 1928, from cercariae of *Bithynia tentaculata* to adults in ducks and *Fulica atra*. According to Odening (1964), the *N. chionis* of the Russian investigators is actually *Notocotylus parviovatus* Yamaguti, 1934. Hsu (1957) reported the development of *Notocotylus mamii* Hsu, 1954, from cercariae of *Melania (Melanoides) tuberculata chinensis* to adults in ducks and guinea pigs. Kupriyanova-Shakhmatova (1959) recalled that L. and U. Szidat (1933) reported that *C. ephemera* Nitzsch, 1807, from *Coretus corneus* developed to adults designated as *Notocotylus thienemanni* and that cercariae from *Galba palustris*, named *C. vaga*, developed into adults designated as *N. attenuatus*. The Russian author fed metacercariae from infections in the two snails and obtained adults that were regarded as specifically identical. Accordingly, *C. vaga* was considered a synonym of *C. ephemera* and *N. thienemanni* as synonym of *N. attenuatus*. If this observation is correct, both *N. thienemanni* and *N. attenuatus* (Rudolphi, 1809) are synonyms of *Notocotylus ephemera* (Nitzsch, 1807) Harwood, 1939. Dönges (1962) reported that cercariae from *Tropidiscus carinatus* are larvae of *Notocotylus ralli* Baylis, 1936. Odening (1963) suggested that *Cercaria monostomi* von Linstow, 1896, is the larval stage of *Notocotylus seineti* Fuhrmann, 1919. Wright and Bennett (1964) found that cercariae from *Lymnaea peregra* develop into *N. attenuatus* when the metacercariae are fed to ducklings, *Anas platyrhynchos*. Zdarska (1964) reported that *C. ephemera* from planorbid snails is the larva of *N. ephemera* and that *C. vaga*

from lymnaeid snails is the larva of *N. attenuatus*. According to this author, the adults differ in number and arrangement of ventral glands and in relative length of metraterm and cirrus sac. Odening (1964) reported that cercariae from *Physa fontalis* (probably *Cercaria monostomi* v. Linstow) developed in chicks to *Notocotylus pacifera* (Noble, 1933) Harwood, 1939. This species, according to Odening, is identical with *N. gibbus* Szidat and Szidat, 1933, *nec* Mehlis in Creplin, 1846. Also, Odening and Bockhardt (1965) found that cercariae from the planorbid snail, *Bathymphalus contortus* (Linn.), developed in the guinea pig to adults of *Notocotylus noyeri* Joyeux, 1922 (syn. *Notocotylus neyrai* Gonzáles Castro, 1945), a parasite of the caecum of European rodents, *Arvicola* spp. Odening (1966) reported the life-cycles of *Notocotylus pacifer*, *Notocotylus ephemera*, *Notocotylus noyeri*, *Notocotylus regis*, *Notocotylus ralli*, and *Catatropis verrucosa*. All employ pulmonate snails as intermediate hosts and all the species of *Notocotylus* have cercariae of the Monostomi type. The cercaria of *C. verrucosa* has an excretory system of the Imbricata type.

These accounts, while somewhat discordant, add materially to knowledge of notocotylid species and provide data that will aid in clarifying the taxonomic problems encountered in the genus *Notocotylus*. The morphology and life-cycle of an additional species, *Notocotylus atlanticus* n. sp., whose asexual generations occur in the brackish-water prosobranch snail, *Hydrobia salsa*, are described in the present paper. Type and paratype specimens deposited in the Helminthological Collection of the U. S. National Museum under the numbers 61,184 and 61,185.

MATERIAL AND METHODS

During the summers of 1963, 1964, 1965 and 1966, over 5000 specimens of *Hydrobia salsa* have been examined for infection by larval trematodes. The snails were identified by Dr. W. K. Emerson of the American Museum of Natural History. This is a somewhat rare, brackish-water species, described by Pilsbry (1905) as *Paludestrina salsa* from Cohasset, Massachusetts. During the summers of 1963 and 1964, the species was common in Nobska Pond, near Woods Hole, Massachusetts, and most of the snails were taken from an area near the connection of the pond with Vineyard Sound. Infection with larval trematodes was heavy and five species of notocotylid cercariae were recognized. In the fall of 1964 the pond was "treated" and most of the invertebrates, including snails, were killed. *Hydrobia salsa* is still absent from Nobska Pond, but in the summer of 1965 the species was found in Oyster Pond, near Falmouth, Massachusetts, and the study of the notocotylid cercariae has been continued. The incidence of infection varied from 0.05% to more than 4%, dependent on the size and age of the snails, time of year and field location. Large snails, in autumn, and from locations where ducks congregate, yielded the highest rate of infection. Incidence was determined by isolation of the snails in groups of ten and, when an infection was observed, by further isolation of these snails. To discover latent infections one snail of each group was crushed, and young cercariae released from rediae were used for study of the development of the excretory system. Cercariae were studied alive and were killed under light coverglass pressure, but the best method for obtaining relaxed specimens of uniform shape is by the addition of hot fixative, AFAG (alcohol,

formalin, acetic acid, glycerine) or the solution of Duboscq-Brasil to a small beaker in which the larvae are being whirled rapidly. The use of vital dyes, Nile blue sulphate and neutral red, facilitated the examination of living larvae.

These notocotylid cercariae are similar in size, shape, and swimming behavior. They swim with the tail in advance, are photosensitive, and accumulate on the light side of the container. As a rule, they emerge between 10 AM and 2 PM and encyst soon thereafter, often within an hour, on the operculum of the snail from which they emerged or other hard surface. Initial feeding of cysts to experimental birds yielded different kinds of adult worms and showed that more than one species was involved. Cysts attached to the shell of a snail may have come from that individual or from other snails.

Specific determination of the infection carried by individual snails is a tedious task, since the snails are small, the cercariae are relatively large and the number liberated is small, two to ten in 24 hours, although an infected snail often will not shed cercariae for several days. The cercariae leave the rediae before they are mature and complete their development in the haemocoel of the snail. The water in the laboratory is warmer than in the ponds; accordingly, in the first days after collection the snails usually shed the cercariae that are mature and some time may elapse before additional cercariae are liberated. Study of the cercariae after emergence provides information on morphology and behavior; sacrifice of the snail ensures that only one species of parasite is present and affords successive stages in the development of the rediae and cercariae. Since identification of encysted metacercariae is difficult if not impossible, specific determination and correlation of larval and adult states were accomplished by feeding metacercariae from individual snails with known cercariae to laboratory-reared hosts. Of the five notocotylid species, two belong to the Yenchingensis Group, two to the Monostomi Group, and the other to the Imbricata Group of cercariae. The present report is concerned with the Yenchingensis cercariae. One species proved identical with *Notocotylus minutus* Stunkard, 1960, and the other is described as a new species, *Notocotylus atlanticus*. Both species develop to maturity in the caeca of eider ducks and *N. minutus* (Fig. 8) has been recovered from experimental infections of domestic ducklings and natural infections of hybrid wild ducks taken off Cuttyhunk Island. The eider chicks were provided by Walter R. Welch and his associates on the staff of the U. S. Fish and Wildlife Service at Boothbay Harbor, Maine, who collected the eggs and hatched the chicks. Their kindness is gratefully acknowledged. Domestic ducklings were purchased from a local hatchery.

DESCRIPTIONS

Notocotylus minutus Stunkard, 1960

Yenchingensis-type cercariae from *H. salsa*, encysted as metacercariae, were fed to domestic ducklings and worms recovered from the caeca agree with those from eider ducks described by Stunkard (1960). Other specimens were found in the caeca of wild hybrid ducks dead at Cuttyhunk Island, frozen and sent to Woods Hole for autopsy. The worms were in poor condition, the ventral glands were indistinct and their number could not be determined. A specimen, shown in Figure 8, is referred to *N. minutus*.

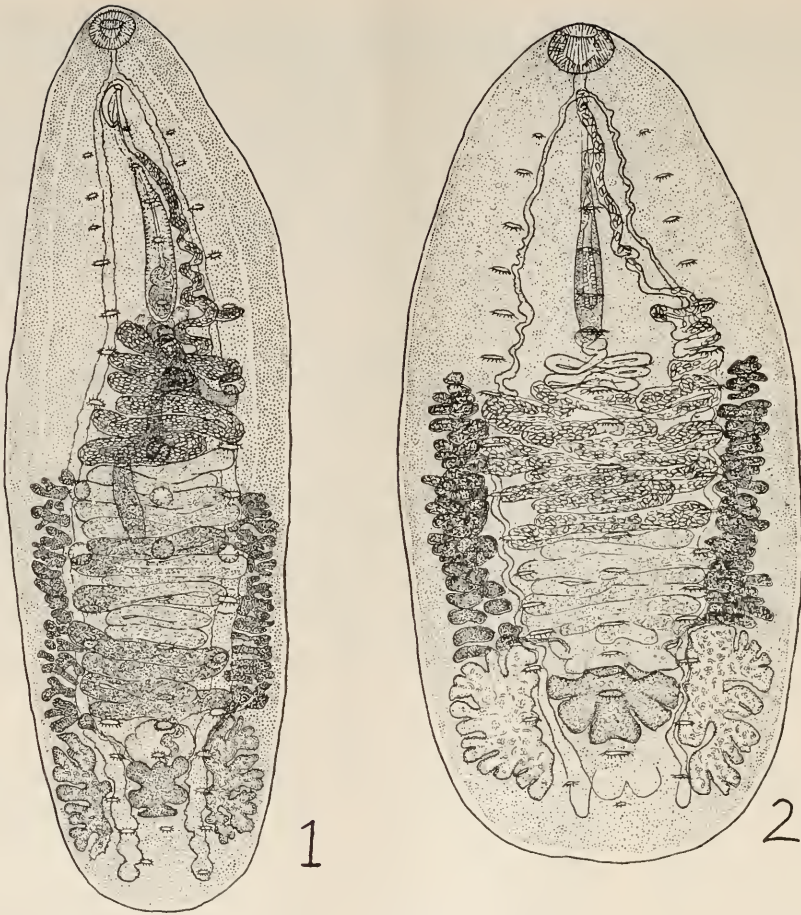


PLATE I

FIGURE 1. *N. atlanticus*, holotype, 3.44 mm. long, from the caecum of an eider duck, experimental infection, ventral view.

FIGURE 2. *N. atlanticus*, paratype, 2.35 mm. long, flattened, from caecum of eider duck, experimental infection, ventral view.

These figures show variation in arrangement of ventral pits and loops of the uterus; also differences in size of organs in a pressed specimen.

Notocotylus atlanticus n. sp.

(Figs. 1-7)

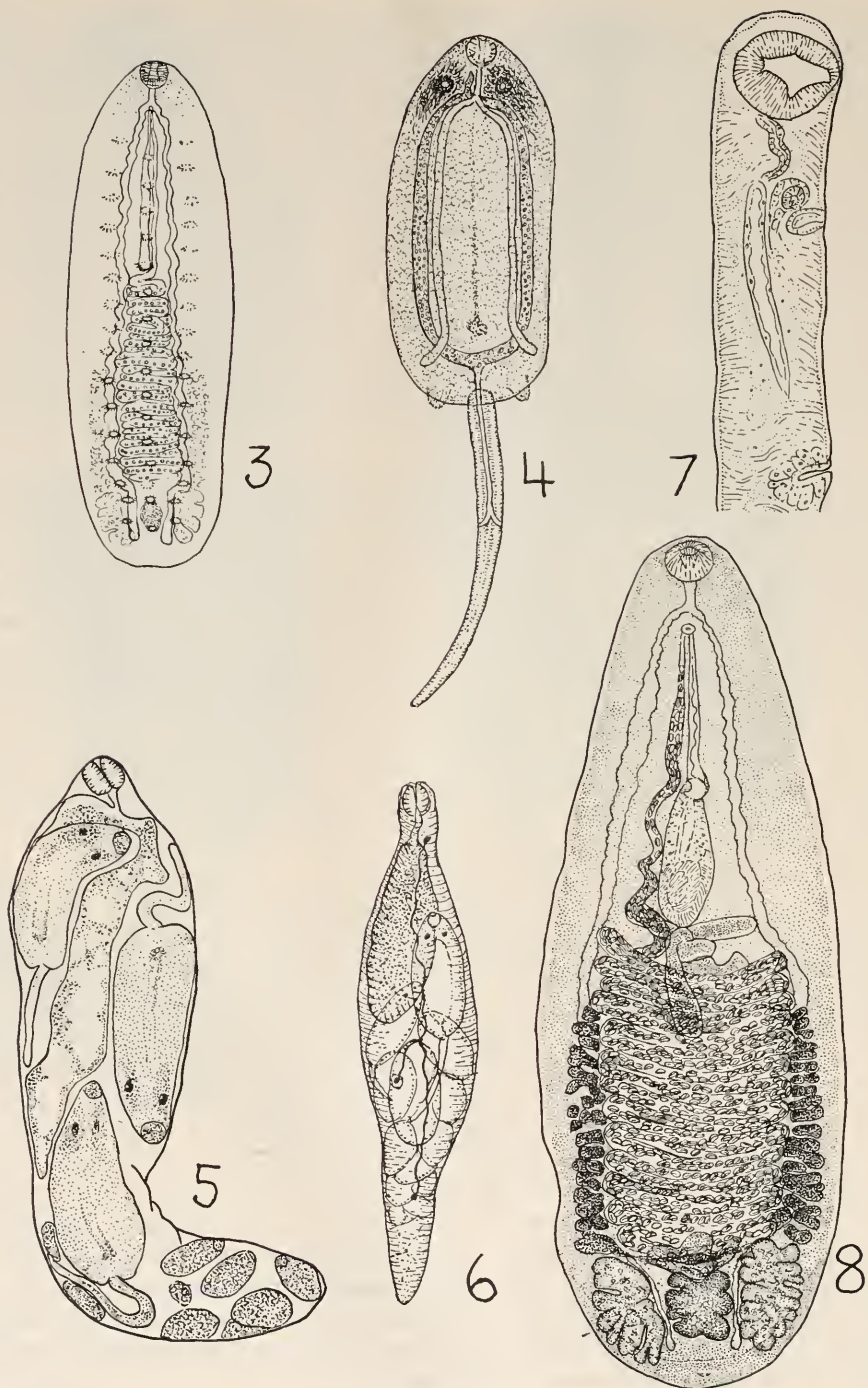
Adult (Figs. 1, 2)

The description is based on ten gravid specimens mounted *in toto* and six cut in serial sections, in sagittal, transverse and frontal planes. The worms are ovate to elongate, flattened dorsoventrally, convex dorsally and concave ventrally. They measure 2.20 to 3.65 mm. in length and 0.80 to 1.10 mm. in width. The cuticula is unarmed, except for the ventral surface, which bears very fine spines, observed

only on living specimens. The anterior portion of the body may bear flecks of pigment from dispersal of the ocelli of the larva, but the pigment disappears in older worms. The body wall is delicate, with the usual circular, longitudinal and oblique muscle layers; the longitudinal fibers are most conspicuous. There are three rows of ventral glands, 16 in each row; those in the median row may be one-half interval anterior or one-half interval posterior to those in the lateral rows. In the specimen shown in Figure 1, the median glands are anterior to the lateral ones whereas in Figure 2, from a much flattened specimen, the lateral glands are anterior. Possibly the arrangement may vary with different states or degrees of contraction of different sets of muscles. The most anterior glands in each row are very small and often hardly recognizable. The oral sucker is circular to oval and measures from 0.15 by 0.12 to 0.18 by 0.15 mm. The esophagus is short and the caeca have irregular lumina. They extend posteriad, turn mediad at the anterior ends of the testes and pass between the testes and ovary, ending blindly behind the level of the gonads.

The testes are lateral, lobed to branched, longer than broad, situated near the posterior end of the body, and measure 0.28 by 0.16 to 0.48 by 0.26. Sperm ducts arise at the anterior ends of the testes, pass anteriad and mediad, uniting just anterior and medial to Mehlis' gland to form the vas deferens which passes forward, dorsal to the uterus. It forms two to four distended irregular coils comprising the external seminal vesicle, which is situated dorsal to the anterior uterine loops, and then enters the cirrus sac where it forms a coiled internal seminal vesicle, 0.25 to 0.375 mm. in extent. The vesicle is followed by a prostatic portion of the male canal and a long ejaculatory duct. The common genital pore is posterior or immediately anterior to the bifurcation of the digestive tract. The cirrus is protrusible and in one specimen measures 0.19 by 0.028. The cirrus sac is 0.60 to 0.84 mm. in length and the posterior end is about three-eighths of the body length from the anterior end. The posterior end of the cirrus sac is situated in the interval between median glands five and six.

The ovary is lobed and measures 0.19 by 0.15 to 0.25 by 0.20 mm. The oviduct arises at the anterior median face and passes anteriad where it receives the common vitelline duct and enters Mehlis' gland. In one series of sections there is a lateral outpocketing of the oviduct, filled with spermatozoa, but a seminal receptacle or Laurer's canal were not observed. The initial part of the uterus is filled with spermatozoa; it coils ventrad and there is a loop of the uterus, with thin-shelled eggs, ventral to Mehlis' gland. There are 12 to 16 transverse loops of the uterus to the level of the anterior lobes of the vitellaria and an additional two to eight loops in front of the vitellaria. The disposition of the uterine loops is dependent on the extension or retractions of the body. The metraterm is less than one-half the length of the cirrus sac; it measures 0.28 to 0.37 mm. in length. The vitellaria are situated in the extracaecal fields and extend from the testes to a level slightly anterior to the middle of the body. Collecting ducts course posteriad along the median faces of the vitellaria, pass mediad around the anterior ends of the testes and unite dorsal to Mehlis' gland to form the common duct that opens into the oviduct. The eggs measure 0.017 to 0.019 by 0.011 mm., and bear long polar filaments. They are embryonated when passed. The excretory system becomes very



FIGURES 3-8.

complex, with many dendritic branches arising from both the lateral and medial faces of the longitudinal trunks of the collection ducts.

Redia (Figs. 5, 6)

The haemocoel of the snail contains many rediae of varying sizes, from very small active individuals, much smaller than cercariae, to large sluggish specimens filled with progeny. A small redia, 0.19 mm. long and 0.048 mm. wide, had a pharynx which measured 0.036 by 0.040 mm., and contained eight germ balls, the largest of which was 0.043 by 0.020 mm. Large rediae may be 1.2 mm. long by 0.14 mm. wide, with a pharynx that measures 0.045 to 0.052 mm. in diameter, and contain four or five cercariae together with a number of germ balls of varying dimensions. Small rediae are colorless, but large ones are orange-yellow in color, with yellow spherules, some 10 microns in diameter, in the body wall. Other yellow droplets and blackish amorphous material are present in the lumen of the intestine. The anterior end bears prepharyngeal lips and short setae. There is a short esophagus, about as long as the pharynx, and a sac-like intestine extends about one-half the length of the body. There are no locomotor appendages. The birth pore is ventral, at the level of the posterior margin of the pharynx. The excretory system is double. The pores are lateral, situated posterior to the middle of the body. From each pore a common duct extends anteriorly for a short distance and then divides into an anterior and a posterior branch, each of which terminates in a flame-cell. The anterior cell is situated near the posterior end of the esophagus and the posterior cell is located about halfway between the excretory pore and the posterior end of the body. The cilia of the flame-cells are 10 to 12 microns long. Each collecting duct is a coiled tube, enclosed in a straight sinus.

Cercaria (Fig. 4)

Living specimens shift from 0.18 to 0.35 mm. in length and 0.11 to 0.19 mm. in width. Fixed in hot killing fluid, they vary from 0.29 by 0.12 mm. to 0.24 by 0.15 mm. The tail is simple, slender; it may be shorter than the body or extend to a length of 0.43 mm., in which condition it is only 0.025 mm. wide at the base. In swimming, the body is contracted, bent ventrally, while the tail is extended and beats violently. Under a coverglass, when the body is extended the tail is contracted and *vice versa*. The tail is subterminal in attachment and in fixed specimens tends to extend at a right angle from the body. The wall of the tail is com-

PLATE II

FIGURE 3. *N. atlanticus*, juvenile specimen, 1.36 mm. long, from caecum of eider duck, 8 days development, ventral view.

FIGURE 4. *N. atlanticus*, cercaria.

FIGURE 5. *N. atlanticus*, redia, 1.00 mm. long, a fixed and stained specimen.

FIGURE 6. *N. atlanticus*, redia from sketches, showing the excretory system of one side; alive it was 0.3 to 0.7 mm. long.

FIGURE 7. *N. atlanticus*, parasagittal section showing relative positions of oral sucker, esophagus, digestive caecum, cirrus sac and metraterm at the genital pore, and most anterior median gland.

FIGURE 8. *N. minutus*, specimen 1.21 mm. long, natural infection, from caecum of hybrid wild duck from Cuttyhunk Island.

posed of external circular and internal longitudinal muscles, disposed around a loose parenchymal matrix. The posterolateral ends of the body bear eversible and retractile locomotor appendages which function in creeping movements. When the body is extended these appendages are close together and they separate as the body contracts. While in the redia, the cercaria has only two ocelli, but after emergence there may be a small, ring-like medial fleck of pigment. The ocelli measure 0.013 to 0.016 mm. in diameter and have lenses which are often conspicuous and give the larva a "spectacled" appearance. The anterior one-third of the body has more or less diffuse, dendritic brown pigment, accumulated around the ocelli, with strands that extend posteriad along the digestive caeca. The oral sucker measures 0.033 to 0.038 mm. in diameter; the esophagus is about as long as the oral sucker and is dorsal to the excretory ring and its anterior projection. The digestive caeca extend posteriad, just mediad of the excretory ring and near the posterior end of the body they turn laterad to cross the ring and end blindly, anterior to the level of the excretory vesicle. In emerged cercariae, the parenchyma is filled with cystogenous cells containing short, bacilliform rods, 2 to 3 microns long and about one-half as wide.

The development of the excretory system of *Cercaria ephemera* Lebour, 1907, was described by Rothschild (1935), with three tufts of actively beating cilia in each recurrent tubule. Miss Rothschild accepted the idea of Faust (1919), that flame-cells increase in number by division, but this postulate has never been confirmed. I have never seen a dividing flame-cell or one with two nuclei, and do not believe flame-cells divide; new cells probably arise from undifferentiated cells in the parenchyma. The system in the cercaria of *N. atlanticus* is virtually identical with that of *C. ephemera*. The primary ducts of the very young cercaria, which extend from the flame-cells to the excretory pores, fuse posteriorly and anteriorly to form the excretory ring; from the locus of anterior fusion a finger-like diverticulum extends forward below the esophagus, and posteriorly from the fused common stem, a tubule leads to the excretory pores on the sides of the developing tail. From the lateral ends of the anterior transverse portion of the ring, recurrent tubules pass posteriad to the middle of the body where they divide into anterior and posterior branches. In young cercariae, before the cystogenous cells become filled with secretion, three flame-cells were observed on each branch and at that stage the system has the formula $2 [(1 + 1 + 1) + (1 + 1 + 1)]$. Odening (1963) reported that the definitive flame-cell formula of notocotylid cercariae is $[(3 + 3 + 3) + (3 + 3 + 3)]$, but his figure of the origin of the secondary tubules from the lateral walls of the ring is not in agreement with present observations. In young cercariae, the constriction that cuts off the tail, delimits a primary excretory vesicle in the anterior end of the common stem; it is immediately posterior to the posterior transverse portion of the ring and in communication with it. As the cercaria matures, a secondary excretory pore is formed from the excretory bladder to the dorsal wall of the body and the portion in the tail atrophies.

Metacercaria

The cercariae emerge principally between 10 AM and 2 PM. They encyst promptly when irritated, otherwise after swimming for two to three hours. When encysting the larva attaches by the oral sucker, contracts until the body is circular,

and rhythmic contractions of the body wall cause the extrusion of the cystogenous material. The tail remains outside the cyst and eventually lashes itself free. As the cyst forms, the external layer remains hyaline, amorphous and jelly-like; as it condenses, a second layer, which has the appearance of flattened, hemispherical disks or droplets, may be recognized, and then a concentric layer of fibrous material encloses the larva. The outside diameter of a newly formed cyst is 0.18 mm.; the inside diameter is 0.16 mm. but the cyst wall becomes thinner, darker and denser with age. The larvae do not develop in the cysts and are infective soon after encystment.

DISCUSSION

The present specimens can be distinguished from species with described life-cycles since, with the exception of *Notocotylus minutus*, all others use fresh-water snails as intermediate hosts. In size, they agree with *N. aegyptiacus*, *N. babai*, *N. filamentis*, *N. noyeri*, *N. seineti*, and *N. dafilae*. But *N. aegyptiacus* and *N. seineti* have fewer ventral glands, *N. babai* has a smaller oral sucker, longer cirrus sac, larger gonads, larger eggs, and a different disposition of uterine loops; *N. dafilae* has a longer cirrus sac and different arrangement of ventral glands and uterine loops, while *N. filamentis* and *N. noyeri* are parasites of mammals with distinct morphological differences. These specimens can not be assigned to any known species and are described as a new species, *Notocotylus atlanticus*.

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OOCYTE DEVELOPMENT AND INCORPORATION OF H³- THYMIDINE AND H³-URIDINE IN PECTINARIA (CISTENIDES) GOULDII

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Transformation from the oogonium and development of the primary oocyte involves intricate morphological and metabolic changes in the cell. Among these are enlargement of the germinal vesicle, development of the nucleolus and cytoplasmic growth (Raven, 1961). It is generally believed that the necessary premeiotic changes in the nucleus precede these phenomena.

Most evidence indicates that H³-thymidine is not incorporated into the germinal vesicle of the mature oocyte (Ficq, 1961a; Favard-Séréno and Durand, 1963b; Ficq, Aiello and Scarano, 1963) although recently Holland and Giese (1965) report that both the oogonia and the pre-leptotene primary oocytes of the sea urchin synthesize DNA within the ovary. These results imply that the synthesis of DNA needed for subsequent growth and maturation of the oocytes must occur after the last oogonial division and no later than the early premeiotic changes in the oocyte nucleus.

Since there is evidence that various types of eggs have vast reserves of cytoplasmic DNA (Bieber *et al.*, 1959) or deoxyribosides (Hoff-Jørgensen and Zeuthen, 1952) and some indication of cytoplasmic uptake (Ficq, 1961a; Gintsburg, 1963) of H³-thymidine into the oocytes, the question arises, is the entire cytoplasmic reserve acquired at the time of nuclear DNA synthesis or is it augmented throughout development of the oocyte? It is pertinent to determine whether the developing and growing oocyte is metabolically stable or if it is able to actively increase its DNA reserve. The continuous presentation of a specifically labeled nucleoside, H³-thymidine, *in vivo* is one approach to this problem.

In contrast, the uptake of RNA precursors into the maturing oocyte appears to be widespread during oogenesis. In the starfish, H³-uridine uptake is localized in the nucleus, nucleolus (Ficq, 1961b) and cytoplasm (Geuskens, 1963), in the nucleus and nucleolus of the sea urchin oocyte (Ficq, Aiello and Scarano, 1963) and the nuclear sap, chromosomes and cytoplasm of the cricket oocyte (Favard-Séréno and Durand, 1963a). The use of H³-uridine and other precursors in conjunction with various inhibitors implicates the synthesis of at least three types of RNA (m-RNA, r-RNA and t-RNA) during maturation of the starfish and sea urchin oocyte (Ficq, 1961a; 1962; 1964).

Since both the nucleolus and nucleus in *Pectinaria* increase in size and activity as the oocyte develops (Tweedell, 1962), a study of the incorporation of labeled uridine into the RNA of the nucleolus and nucleus seemed promising (Tweedell, 1964).

Fortunately, the growth and development of the primary oocyte in *Pectinaria* occurs in the coelom after the cells leave the ovary. The utilization of these labeled precursors can be observed *in vivo* within the ovary and during individual stages of oocyte formation in the coelom. These results are compared with *in vitro* exposure of the oocytes to the same nucleosides.

MATERIALS AND METHODS

Specimens of the marine polychaete annelid *Pectinaria* (= *Cistenides*) *gouldii* Verrill (Hartman, 1941) were obtained from the north shore of Cape Cod during June, July and August of 1963 and 1964. They were maintained in the laboratory as described earlier (Tweedell, 1962).

Incorporation of isotopes

In vivo. Before labeling, the animals were carefully removed from their sand test and the sex was determined microscopically by identification of the gametes beneath the ventral body wall or the parapodia. The animals were then narcotized with 50% ethyl alcohol added dropwise to a small dish of sea water until the animals were flaccid. Isotopes were injected with a #27 hypodermic needle directly through the cephalic plaque (Fig. 1) of the animals into the coelomic cavity. Narcotized animals were injected according to size with either 5 to 10 μ C. of tritiated thymidine (1.9 C./millimole) or 5 to 10 μ C. of tritiated uridine (1.7 C./millimole). The addition of a small amount of Nile blue sulphate indicated that most of the injected fluid was retained. The animals were then returned to their tests and placed in normal sea water where they recovered quickly. Animals were sacrificed or the eggs were harvested and fixed at intervals of $\frac{1}{2}$, 1, 2, 4, 8, 16, 24, 48 and 72 hours. Additional egg harvests were made at 2, 3, 6-7 and 19-21 days after injection. At least two animals were examined for each of the above intervals.

In vitro. Gametes were also shed directly into sea water containing 10 μ C. of H^3 -thymidine or H^3 -uridine per ml. of sea water. Following germinal vesicle (G.V.) breakdown of the mature oocytes, they were removed, washed and fixed at intervals of 2, 4 and 8 hours after exposure. Thereafter they were embedded in paraffin and sectioned at 4 μ .

Recovery of free oocytes. Some of the animals injected *in vivo* were shed and the eggs immediately fixed as whole mounts on coverslips with Kahle's fixative using a modified double coverslip sandwich technique (Tweedell, 1962). Similar treatment was given to oocytes which were labeled by shedding directly into sea water containing the isotopes. At the moment of fixation, the cells were compressed by placing 45 grams pressure on the coverslips to further flatten the oocytes. After fixation the whole mounts were washed and dehydrated to 100% ethyl alcohol when they were air dried. The coverslips were then cemented to slides with the eggs facing upward. Equivalent animals were fixed at the same time for future exposure of the oocytes in sections.

Preparation of intact animals. The majority of the oocytes labeled *in vivo* were processed for sectioning *in situ*. Prior to histological preparation of adult animals it was necessary to remove the sand grains from the digestive tract. Orig-

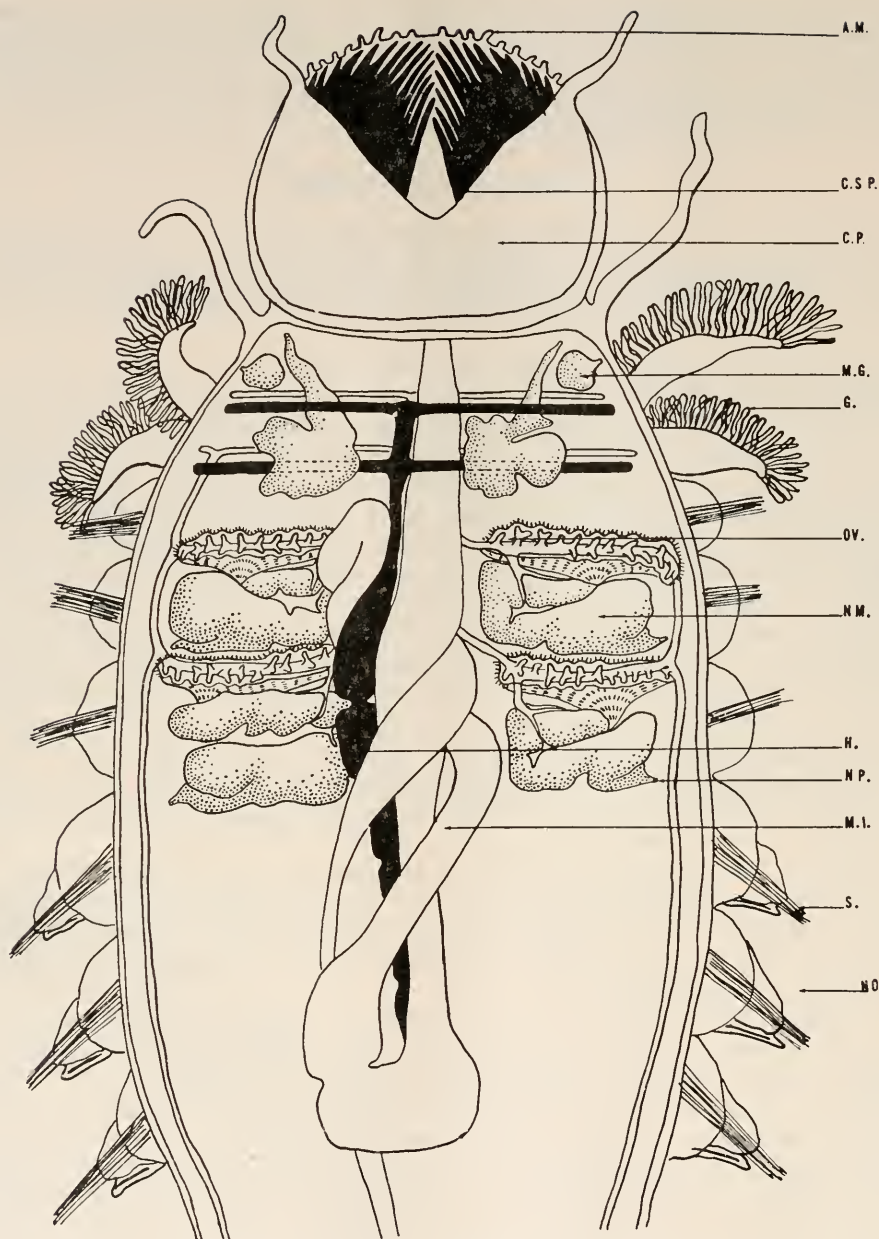


FIGURE 1. A dorsal dissection, cut in the midline and spread laterally, of an adult *Pectinaria* (♀), showing position of the ovaries and attached nephromixia. CSP, cephalic spines; CP, cephalic plaque, AM, antennular membrane; G, gill; NM, nephromixium; NP, nephrodio-pore; MG, mucus gland; OV, ovary; NO, notopodium; S, setae; MI, middle intestine; H, heart.

nally, the technique of Hanks (1960) was repeated, in which he fed living animals ground quahog shells. As reported, the animals readily ingested the calcium carbonate. However, the required decalcification by acetic acid in the *intact* animal was not successful and was further complicated by the collection of CO_2 which could not be removed under vacuum. Alternate materials, dried coffee grounds, corn meal, powdered charcoal, ground rice, Dowex resin and various household cereals were tested. In all cases, the materials were ground and screened through wire mesh to the equivalent size of large sand grains. These materials were placed in 5-inch open glass tubes, $\frac{1}{2}$ -inch diameter, that were covered at one end with several layers of cheesecloth. The tubes were half-filled with the test material and placed cloth end down in a test tube rack. The adult animal was inserted head down in the tube and a siphon tube of slowly running sea water was introduced at the top to insure a constant sea water flow. Animals were allowed to "feed" in this position for 24 to 48 hours, then removed and left to "work" in running sea water for 24 hours while excess ingested products were eliminated. Three materials were successful: powdered charcoal, ground white rice grains and a protein cereal (Kellogg's Concentrate). The latter two were readily ingested and perfectly compatible with histological sectioning while the charcoal sections tended to crumble. After removal from their tests the adult animals were narcotized and then fixed in Kahle's or Kleinenberg's fixative, double embedded in methyl benzoate-parlodion paraffin and sectioned at 5 μ .

Autoradiography. Both compressed whole oocytes and sections were prepared for autoradiography by dipping the slides in Kodak NTB2 or Ilford G-5 emulsions (diluted 50/50 with distilled water). The slides were incubated in light-tight boxes with "Drierite" at 4° C. for the duration of exposure from two to three weeks. Slides were developed in D-72 or D-19 for 3 minutes at 14° C., placed in stop bath for 15 seconds and fixed for 3 minutes at 14° C. The slides were then washed for $\frac{1}{2}$ hour in running water.

Coverslip mounts were stained in gallocyanin chrome-alum, pH 1.7, for 8–12 hours and counterstained with eosin. Sections were stained in Galligher's haematoxylin, eosin-azure II (Gurr) or Jenner-Giemsa solution.

Serial sections of oocytes retained within the whole animal or sections of shed eggs were treated with RNase or DNase prior to autoradiography. DNase was applied to sections alternating with control sections in a concentration of 0.1 mg./ml. prepared in 0.05 M Michaelis veronal acetate buffer, pH 6.8 with 0.0025 M MgCl_2 added. Incubation was at 37° C. for 2 hours.

RNase was prepared as 3 mg./ml. in 0.05 M Sorensen phosphate buffer at pH 7.6. The sections were incubated in RNase for 2 hours at 48° C., along with non-enzyme-treated control sections.

OBSERVATIONS

The ovaries are minute, well vascularized organs located along the anterior surface of two pairs of prominent yellow brown organs, identified as nephromixia (Goodrich, 1945). Externally, the paired nephromixia can be seen through the lateral body walls of the 2nd and 3rd setae-bearing segments. The latter glands, attached to the ventral body wall (Fig. 1) first loop dorsally at the midline, then

open externally through dorso-lateral nephridiopores which are slightly caudal to the abbreviated setae of the parapodia on the same segments.

Viewed from a dorsal dissection, the ovaries are seen anterior and slightly dorsal to the nephromixia. The ciliated ovaries are narrow transparent organs enveloping lateral ovarian blood vessels which flow along the cephalic edges of the nephromixia. Lobe-shaped fins projecting at right angles to the main axis of the ovary extend dorsad and somewhat anteriorly from the ovaries. These fins are sinuses confluent with the ovarian vessel and are also covered with cilia.

The germinal portion of the ovary consists of budding areas located at the ventral medial end of each of four ovaries (Figs. 2, 3). Sometimes an additional area is found at the dorsal lateral end. The ovarian cytology seems to vary according to the season. Early in the summer the ovaries are largely composed of inter-phase oogonial cells and mitotic figures are common, whereas in August the ovaries have a large proportion of oocytes in the leptotene stage (Fig. 4).

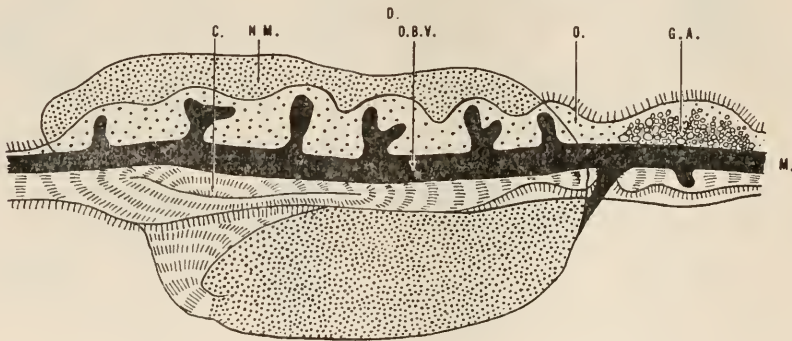


FIGURE 2. An anterior view of a single nephromixium and attached ovary (as seen in Figure 1). D, dorsal; M, medial; C, coelomostome; NM, nephromixium; OV, ovary, enclosing lateral ovarian vessel; GA, germinal area.

The generative phase (Raven, 1961) of oocyte formation begins within the germinal area of the ovary. The oocytes are budded off directly into the coelomic cavity from the germinal area at the cephalic free edge of the ovaries. Thus a sample of the coelomic fluid from an adult female yields a series of developmental phases in the formation of the primary oocytes after they leave the ovaries. The developing oocytes are moved and bathed continuously in this fluid by the muscular pumping of the adult. A diverse selection of cell types: amoebocytes, gregarines, histiocytes and others, are included in the coelomic fluid.

Two development phases of oocyte formation within the coelom can be recognized, a packet phase that is followed by an individual oocyte (solitary) phase. The packets consist of small cell clumps, about 18 to 20 microns in diameter, arranged about a central core area. They appear in the coelomic fluid after being budded from the surface of the ovary (see Fig. 3).

There usually are 16 to 32 oocytes within a packet and the individual cell is about 5.5 microns in diameter. The nucleus fills the cell, leaving a thin rim of cytoplasm, and the chromosomes are generally in the zygotene stage. The single

nucleolus is about twice the size of those within the ovary. Thereafter the packets slowly increase in size from growth of the individual cells and a small "core" forms in the packet center. The enlarged nuclei, entering the pachytene stage, now have a dominant nucleolus and the cytoplasm shows heavy basophilia (stainable with alcian blue, galloxyanin and toluidine blue) at the basal ends of the cells near the center of the packet. The largest packets consist of loosely bound cells each measuring 10 to 20 microns in width.

During the second growth or vegetative phase, the packets fragment and individual oocytes appear in the coelomic fluid, the smallest measuring from 10 to 12.5 microns. These cells are distinguished by a large germinal vesicle and a single amphinucleolus. The increase in size of these solitary oocytes is due to both nuclear and cytoplasmic growth. The mature oocyte is a flattened ellipse which averages 48 by 56 microns and, since it is flattened, about 25 microns in thickness. At this time the germinal vesicle has an average diameter of 40 microns. Thus, using the cell diameter as a crude measurement of linear growth, the cells are seen to increase about ten times in size since budding from the ovary.

As the oocyte approaches full development, the diplotene chromosomes are conspicuously distributed throughout the germinal vesicle. Increased activity of the nucleolus is evidenced by the formation of epinucleolar and intranucleolar buds.

Mechanical stimulation of the adult usually causes artificial shedding when part or all of the coelomic fluid with its cellular components is ejected from posterior coelomoducts. Approximately 12 to 18 minutes after shedding into sea water (Austin, 1963; Tweedell, 1962) the fully developed oocytes undergo germinal vesicle breakdown, become spherical and develop to the metaphase of the first maturation division where they remain unless fertilized.

There are indications, however, that the natural spawning process follows a different sequence of events. During late summer induced shedding occasionally produces oocytes that are already in the first maturation division. However, these cells are ejected from the nephridiopores. Ordinarily, the nephromixia are relatively empty, but during late summer their distal ends contain heavy concentrations of oocytes. Coelomic oocytes are drawn into the nephromixium along a heavily ciliated groove that lies on its anterior edge. The ciliary action transports the oocytes laterally to a large ciliated funnel (coelomostome) through which they enter the nephromixium (Fig. 2). Upon stimulation, these oocytes are readily observed leaving the nephridiopores.

In addition, sections through the intact animal reveal gravid nephromixia in which the enclosed oocytes are almost exclusively in a post-G.V. state while the germinal vesicles of fully developed oocytes within the coelom are still intact (Fig. 5). Presumably, during the natural spawning process G.V. breakdown occurs within the nephromixia and the oocytes are shed through the nephridiopores.

Some of the cells within the nephromixium after germinal vesicle breakdown are often still in late diakinesis (Fig. 6). Chromosome bivalents are widely spread throughout the cytoplasm and the nucleolus is absent. Generally it is possible to distinguish 16 large bivalents, yet as many as 9 additional obscure, weakly staining doublets can be seen. Austin (1963) reports from 17 to 21 bivalents from his observations, the variation being attributed to faint, minute chromosomes. Obviously a detailed karyotype study is needed. In other cells within the nephro-

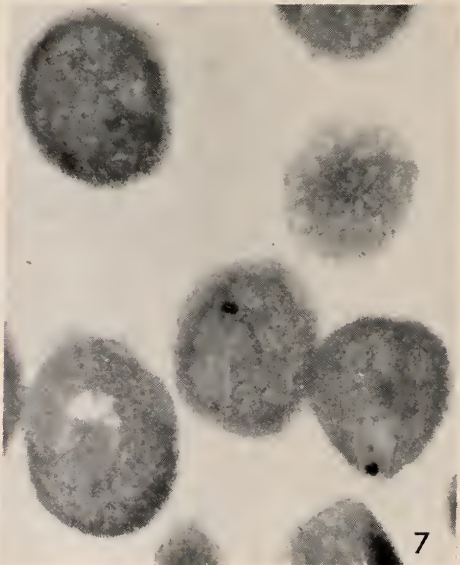
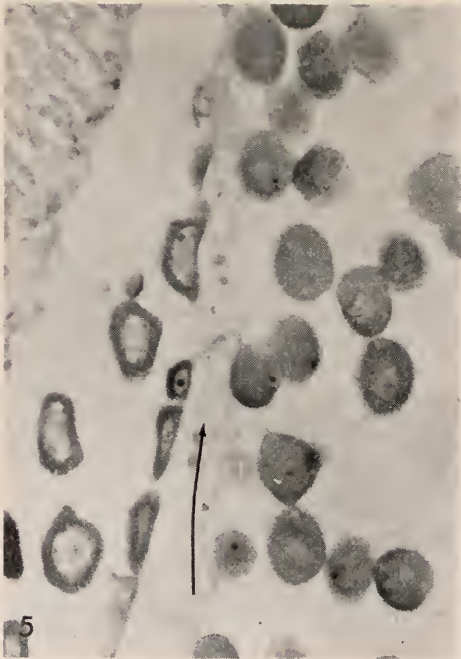
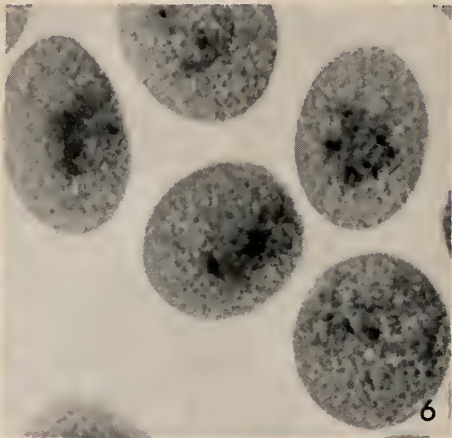


PLATE I

nixium, the chromosomes are condensed into a small knot, where they remain arrested in the first maturation division.

RESULTS

H³-thymidine

In vivo labeling. Following injection of tritiated thymidine into the living adult, oocytes were harvested by shedding and prepared as whole mounts or embedded and sectioned. Entire animals were also embedded and sectioned intact.

The ovary. Tritiated thymidine was quickly incorporated into the ovary after injection into the coelom. Localization was principally over the nuclei of cells scattered throughout the ovary. The first uptake was detected two hours after contact with H³-thymidine (Fig. 8). The labeled cells were generally found deep in the mid region of the ovary rather than at the free surface. Since the label in the ovary often appeared above the nuclei of cell pairs, these cells presumably had just completed cell division and the resultant oocytes were exposed to thymidine during the period of DNA synthesis (S period). Oocytes associated with the budding surface of the ovary did not show appreciable nuclear label at this time.

After 12 hours exposure to the thymidine some of the deep-lying cells of the ovary were intensely labeled while those along the surface were more lightly marked (Fig. 9). Scattered labeled cells appeared throughout the ovary and extended to the free border where they budded off. Labeled oocytes were also observed in ovaries recovered from animals 24 and 48 hours after injection of H³-thymidine with no essential difference.

Heavy nuclear labeling was still evident in ovarian oocytes taken from animals after one-week exposures. The major change from earlier ovarian sections was the presence of more labeled cells at the free surface of the ovary (Fig. 10). Recently released labeled oocytes were often seen adjacent to the ovary. In general the labeled oocytes were more widely distributed but less heavily tagged over the nuclei.

After 21 days exposure to a single pulse, grains were diffusely scattered over the remaining intact oocytes. This distribution suggested that the incorporated thymidine had been diluted out by subsequent mitoses of the remaining oogonia.

The actual length of the synthesis period prior to oocyte growth and development was undetermined but H³-thymidine incorporation in the ovary did indicate when DNA synthesis took place. The assumption was then made that the cells which incorporate thymidine after a short pulse time were either oogonia or pre-

PLATE I

FIGURE 3. Cross-section of a normal ovary and attached ciliated lobe. Oocytes are budding from free edge of ovary at lower left.

FIGURE 4. Developing primary oocytes in premeiotic stages within the ovary. 1920 ×.

FIGURE 5. Post-G.V. oocytes within lumen of nephromixium on the right; pre-G.V. coelomic oocytes to the left. Arrow indicates wall of nephromixium separating pre- and post-G.V. oocytes. H³-thymidine-injected, 2-hour exposure. No uptake in either pre- or post-G.V. oocytes. 350 ×.

FIGURE 6. Post-G.V. oocytes from within nephromixium of control animal. Optical plane shows portion of chromosomes in diakinesis. 1200 ×.

FIGURE 7. Post-G.V. oocytes in nephromixium showing absence of H³-thymidine incorporation after a two-hour pulse. 580 ×.

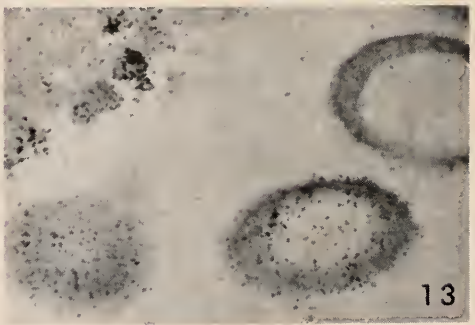
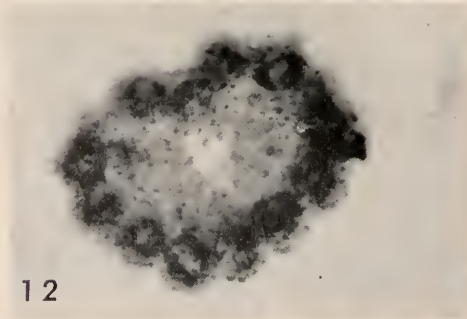
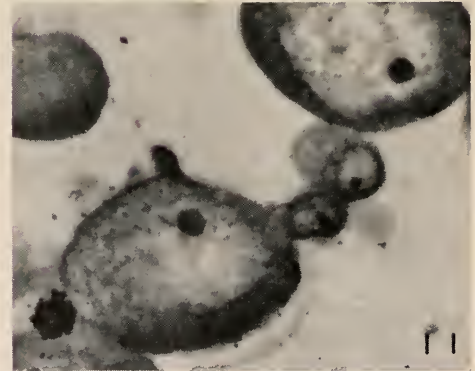
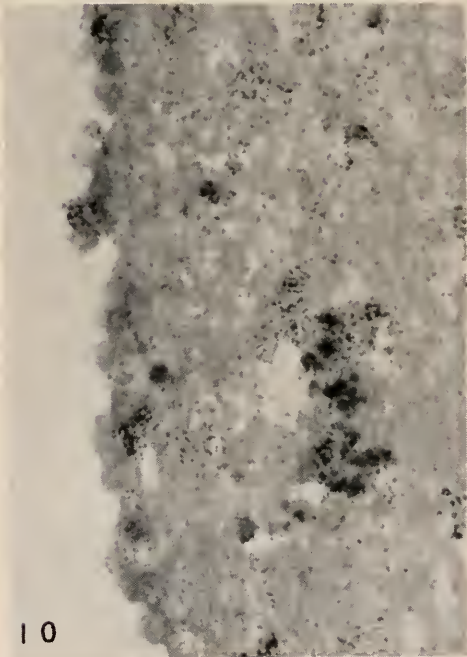
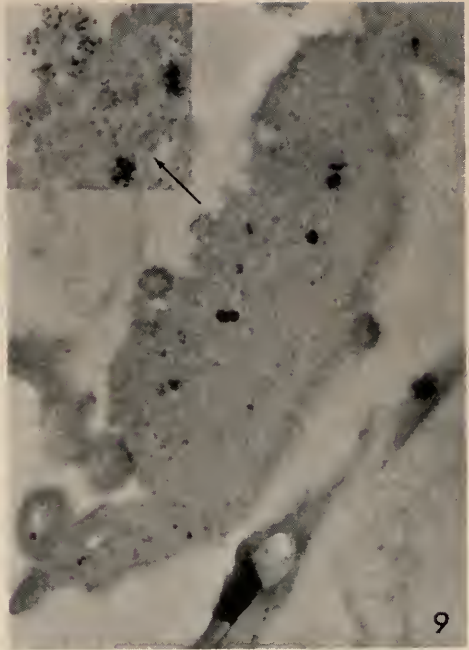
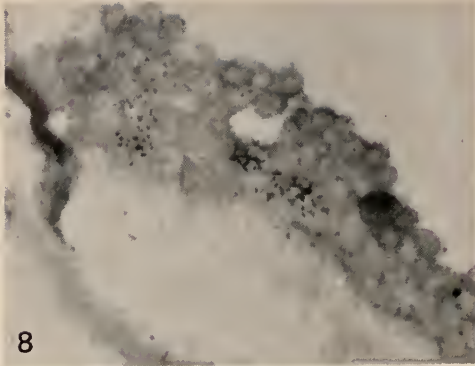


PLATE II

meiotic oocytes in the "S" period preceding growth and development of the oocyte. Consequently, the developmental age of subsequent stages found with nuclear label was extrapolated at successive intervals after injection; the next most advanced stage so labeled indicated its developmental age.

Coelomic oocytes. After a short exposure period, thymidine was also readily incorporated into the oocyte packets of the coelom. The especial difference between the ovarian oocytes and the oocyte packets was in the localization of the radioactivity. The packet oocytes were mainly labeled above the cytoplasm with relatively little nuclear label.

The smallest coelomic oocyte packets, containing cells of a size comparable to ovarian oocytes, began showing a label primarily over the cytoplasm after a pulse of 30 minutes. Thereafter, recovery of shed oocytes from animals at intervals of 2, 4, 8, 16 or 24 hours after injection revealed the same pattern of labeling. An example of a packet recovered after 24 hours from an animal injected with 5 μ C. is seen in Figure 11. The nuclei of these small packet cells were in the zygotene stage. Larger cell packets and single oocytes were noticeably unlabeled. Sections of oocytes *in situ* from a parallel series of injected animals demonstrated identical uptake in the very early packets of oocytes recovered up to 24 hours after injection.

As it was not possible to effectively chase the tritiated thymidine from the coelom of the living animal, it was not certain whether these early cell packets incorporated the thymidine before or after leaving the ovary. The latter possibility was most likely since ovarian oocytes at the budding edge of the ovary did not become labeled until after 12 hours injection. Thus, these small packets were distinctive since they were the only coelomic oocyte stages found with a significant grain count after 2 to 24 hours exposure to tritiated thymidine.

Examination of coelomic oocytes sectioned *in situ* after an exposure of two days indicated a significant change in the labeling of cell packets. A few larger oocyte packets, presumably resulting from cellular growth of younger packets, began exhibiting a heavy cytoplasmic label with very few grains over the nucleus. The additional labeling of older oocyte packets suggested that the developing oocytes had acquired the H^3 -thymidine during the early packet stage and that cytoplasmic and nuclear growth of the individual oocytes followed subsequently.

Both small and medium-size oocyte packets continued to show a predominately cytoplasmic label up to 72 hours after exposure to labeled thymidine (Fig. 12); others had a light nuclear label.

PLATE II

FIGURE 8. Edge of ovary after a two-hour exposure to H^3 -thymidine. Label occurs over isolated cells along the interior.

FIGURE 9. Ovary from H^3 -thymidine-injected animal sacrificed after 12 hours. Scattered oocytes are heavily labeled over the nuclei. The free budding edge of ovary is at the left. A small labeled oocyte packet is seen in insert. 234 $\times$.

FIGURE 10. A section of ovary from a H^3 -thymidine-injected animal sacrificed after 7 days, showing scattered distribution of labeled oocytes. 585 $\times$.

FIGURE 11. Single coelomic oocytes which are unlabeled after a two-hour pulse of H^3 -thymidine. A heavily labeled oocyte packet is seen at the lower left. 375 $\times$.

FIGURE 12. A medium-size packet of developing oocytes from an animal sacrificed 72 hours after H^3 -thymidine injection. Incorporation is principally over the cytoplasm.

FIGURE 13. Large single developing oocytes recovered 7 days after H^3 -thymidine injection. A heavy label finally appears over the nucleus and cytoplasm. The edge of the ovary appears on the left. Note that *not* all of the large oocytes are labeled. 585 $\times$.

After a prolonged exposure of 7 days, many small and medium oocyte packets were labeled as they had been earlier. More significantly, new oocyte packets with a heavy nuclear label appeared. Since cell division does not occur until germinal vesicle breakdown, these packets must have been labeled and subsequently released from the ovary.

Animals which had been injected with H^3 -thymidine were also sacrificed after 21 days. Both small and medium cell packets and a few late oocyte packets were found with evidence of thymidine uptake. In the small packets the grains were scattered over the cytoplasm but the heaviest concentration occurred over the nucleus. These packets possibly represented cells that had taken thymidine into the nucleus at an earlier time and the labeled material had moved into the cytoplasm secondarily. Alternate oocyte sections from one animal exposed *in situ* for 21 days were pretreated with DNase prior to emulsion dipping. The DNase removed both the nuclear and cytoplasmic label from the oocyte packets.

Single oocytes. Individual coelomic oocytes in the final growth phase failed to indicate any incorporation of H^3 -thymidine whether they were harvested early ($\frac{1}{2}$ to 16 hours), as seen in Figure 11, or after prolonged exposure (1 to 2 days). The same results were obtained from compressed egg whole mounts and from a study of sections of 8 entire animals sacrificed at 2, 12 or 24 hours after injection with either 5 or 10 μ C. of tritiated thymidine. The solitary, small immature coelomic oocytes were completely unlabeled in all material harvested up to a day after injection. The larger mature cells also failed to take in the precursor. It was also found that mature oocytes, in metaphase I, within the nephromyxium showed a complete absence of any labeling (Fig. 7).

Occasional single oocytes, in their early growth phase, were not significantly tagged until 48 to 72 hours after injection. In these cases the grains were evenly distributed over the entire cell. The majority of these single oocytes, however, remained unlabeled. A sampling of 50 fields from different slides showed an average of 9% of the larger, single oocytes were tagged at 72 hours. The average number of grains per labeled cell was 196 as compared to a background count of 7 from an equivalent area.

Since even the smallest single oocyte failed to show uptake of thymidine after a pulse of 30 minutes, it was assumed that the small number of older oocytes were labeled at the packet stage or earlier. Therefore, the minimal time for an oocyte to reach full size appeared to be from 48 to 72 hours.

Isolated single oocytes, recovered at still longer periods after injection gave further evidence of thymidine redistribution. A study of three different H^3 -thymidine-injected animals sacrificed and sectioned 7 days after injection showed again that individual maturing oocytes were labeled. The marked cells were few and scattered, and the grains appeared with equal intensity over the entire cell. In many cases a heavily labeled oocyte occurred adjacent to a group of entirely unlabeled mature oocytes (Fig. 13). Sections through the center of the oocyte indicated little if any of the label was concentrated in the nucleus. This also suggested that the radioactive material had entered the cell during an earlier packet phase.

In animals sacrificed after 21 days individual oocytes of increasing sizes were also tagged. Many of the larger oocytes were still unlabeled but a survey of 50

fields indicated that an average of 17% of the largest oocytes had the labeled precursor. The grain count averaged 228 per cell compared to 9 for an equal area of background. It was apparent that some of these oocytes approaching maturity were more lightly labeled than others. The grains were distributed evenly over the nucleus and the cytoplasm in the more heavily labeled cells. Some of the lighter marked cells showed grains only over the cytoplasm.

In vitro labeling. The previous results demonstrated the inability of solitary developing coelomic oocytes or mature oocytes to incorporate H^3 -thymidine directly into the nucleus but indicated a possible cytoplasmic uptake of thymidine. Conceivably, the events associated with the breakdown of the germinal vesicle and entrance of the cell into metaphase I could have altered the pattern of thymidine incorporation; this seemed to justify further tests on the oocytes after G.V. breakdown.

Direct shedding of the oocyte stages into H^3 -thymidine provided a more concise measure of the time of uptake into the early packet stages. The absence of the ovary assured that the earliest stages were post-ovulatory and relatively short pulse times could be tested after adequate washing, assuring that appearance of a label over a particular stage would represent actual uptake at that developmental stage.

Oocytes were shed directly into sea water containing the precursors, and by regulating the time of initial exposure to the labeled compounds, it was possible to compare the mature primary oocytes before, during and after G.V. breakdown. Initial exposure to the labeled compounds was made from 5 minutes after shedding (prior to G.V. breakdown) and up to one hour post-shedding (after G.V. breakdown). The cells were usually pulsed for two hours but in some cases were left in the isotopes for 10 to 12 hours.

Oocytes placed into 5 or 10 $\mu C.$ of thymidine/ml. of sea water between 5 and 10 minutes after shedding always possessed intact germinal vesicles at the time they were introduced. After one or two hours they were washed, fixed and prepared as whole mounts or embedded and sectioned.

In some cases G.V. breakdown did not occur in any of the full grown oocytes but this was attributed to a lack of maturity. In other experiments up to 80% of the cells showed G.V. breakdown. In neither instance did any of the single oocytes show any nucleoside incorporation. Prolonged exposure of the oocytes for 10 to 12 hours had no effect.

Oocytes were also shed into plain sea water, where mature oocytes underwent G.V. breakdown. After 20 minutes they were placed into sea water containing H^3 -thymidine. The results were the same. None of the post-G.V. oocytes showed any thymidine incorporation.

At the same time the smaller oocyte packets harvested after two hours exposure showed moderate to heavy labeling over the cytoplasm, confirming what had been found with *in vivo* labeling. None of the packets exhibited any significant nuclear label, which verified the earlier observations that nuclear labeling was confined to cells within the ovary.

In order to substantiate the nature of the thymidine uptake in the cytoplasm, sections of oocytes which had been pulsed for three hours were exposed to DNase

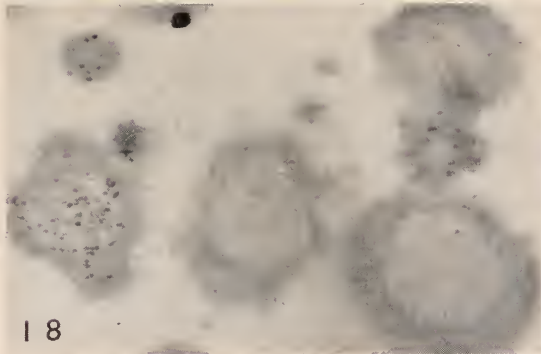
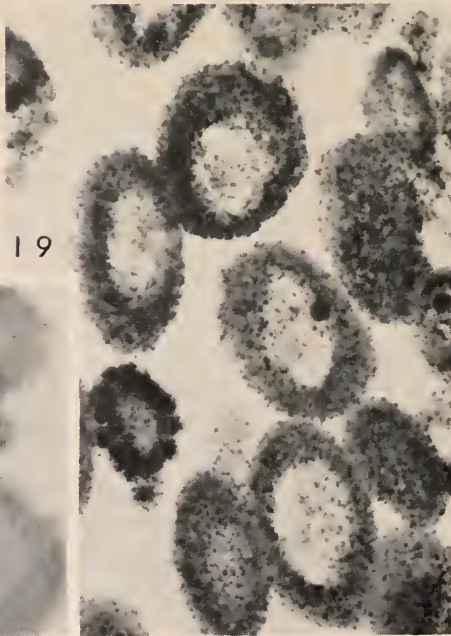
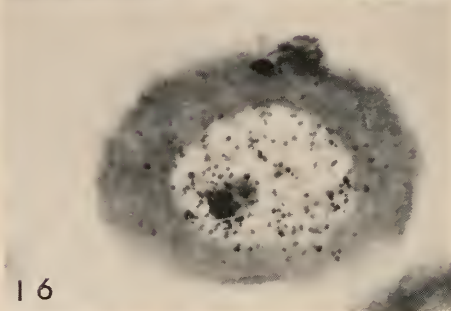
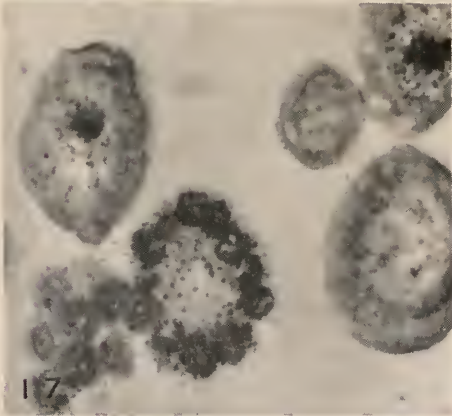
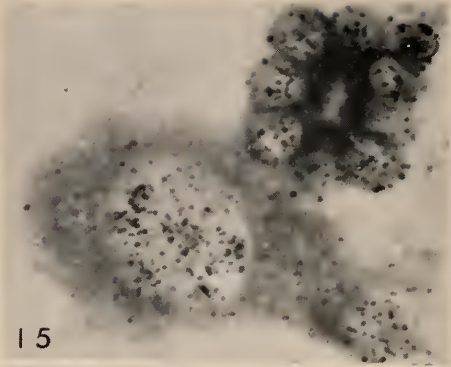
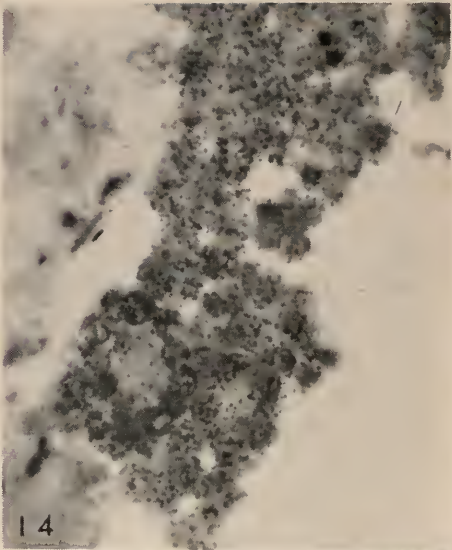


PLATE III

prior to dipping in the emulsion. The DNase treatment completely eliminated any labeling in the cytoplasm of the young oocyte packets.

H^3 -uridine

In vivo labeling. Animals were pulsed with either 5 or 10 μ C. of H^3 -uridine, then the eggs were shed and sectioned or sectioned *in situ*.

The ovary. Unlike the uptake of thymidine in the ovary, H^3 -uridine incorporation was evenly distributed throughout the ovary. As soon as 4 hours after injection, the ovary was uniformly labeled over the entire area. Uridine appeared to be taken up equally in the nuclei and cytoplasm of the ovarian cells. The intensity of the label increased with the exposure time so that after 24 hours the ovary exhibited a dense, diffuse distribution of silver grains. Ovaries from animals sacrificed after 48 to 72 hours exposure presented a heavy nuclear label as well as a moderate concentration of grains over the cytoplasm of ovarian oocytes (Fig. 14). After prolonged exposures of 6 days the ovary was still heavily labeled, particularly over the cytoplasm of the oocytes nearest the periphery, while the nuclear concentration had diminished.

Coelomic oocytes. All stages of coelomic oocytes exhibited moderate to heavy nuclear uptake of radioactive uridine after short pulses. While the initial labeling was nuclear, the distribution of the precursor changed with increased exposure.

Oocyte packets. Oocytes exposed to H^3 -uridine in the early phase of packet development possessed a moderate to heavy concentration of grains over their nuclei after two hours (Fig. 15). Medium-sized packets were also labeled primarily over the nucleus although a light scattering of grains also appeared over the peripheral or outer cytoplasm. The basal ends which stain heavily for basophilia of the oocytes directed toward the packet center demonstrated little uptake of uridine.

After a 4-hour exposure, the nuclear label became greatly intensified and the cytoplasm accrued additional grain accumulation. Small compact oocyte packets with prominent nuclear labeling also showed diffuse scattered grains, particularly around the peripheral cytoplasmic areas. This same condition was found in oocyte packets after 8 and 24 hours exposure to the uridine. Significantly, in oocyte packets of larger sizes, the granular label was found over the nuclei with scarcely a trace in the cytoplasm.

It was very apparent that the cytoplasmic label first detected in the smaller oocyte groups was intensely distributed over all cell packets, except the large ter-

PLATE III

FIGURE 14. Portion of the ovary with diffuse labeling of oocytes 72 hours after H^3 -uridine injection. An oocyte packet is superimposed. 750 $\times$.

FIGURE 15. Oocyte packet with strong nuclear and light cytoplasmic label adjacent to an individual oocyte. Shed two hours after H^3 -uridine injection. 960 $\times$.

FIGURE 16. Single oocyte with labeled nucleus and heavy concentration of grains over nucleolar bud. Two-hour H^3 -uridine pulse. 960 $\times$.

FIGURE 17. A comparison of the heavy cytoplasmic label in oocyte packets with the predominately nuclear and nucleolar uptake in single oocytes. Sacrificed after 72 hours H^3 -uridine injection. 936 $\times$.

FIGURE 18. Coelomic oocytes after two-hour pulse of H^3 -uridine and subsequent treatment with RNase. Most cells are unlabeled but occasional nuclei resist treatment and show retention of label. 936 $\times$.

minimal oocyte packets, after 48 hours exposure to H^3 -uridine. Presumably, the persistent appearance of the cytoplasmic label in these oocytes either represented utilization of H^3 -uridine by RNA or its precursors already in the cytoplasm, or resulted from the movement of RNA from the nucleus into the cytoplasm.

Oocytes in early packet stages continued to display heavy cytoplasmic and nuclear label when the animals were sacrificed 72 hours after injection. Both small and intermediate oocyte packets were affected alike, as seen in Figure 17. Again, in the transition to larger cell packets the grains were still limited to the nuclei with little cytoplasmic concentration.

Single oocytes. After a short pulse (15 to 30 minutes) single cells also gave evidence of H^3 -uridine uptake. Two hours after being injected and shed, the smaller single oocytes appeared with a heavy nuclear label (Fig. 15) while larger oocytes showed a less dense nuclear labeling as seen in Figure 16. In some cells sacrificed after 4 hours the nuclear and nucleolar label was so intense that the cells appeared almost black in these areas. There was often a concentration of grains around the nucleoli, particularly above the epinuclear buds (Fig. 16). The cytoplasmic label of scattered single oocytes varied from light to moderate.

Single oocytes which had been shed and recovered 8 hours after injection of the adult animal gave the same pattern. Medium to large single oocytes were heavily tagged over the nucleus, nucleolus and somewhat over the cytoplasm. In the less intensely tagged cells, the nuclei were principally labeled.

Following 24 hours exposure to H^3 -uridine, preparations of sectioned oocytes *in situ* indicated that all sizes of single oocytes had a strong nuclear incorporation with scattered grains over the cytoplasm. A survey of 50 fields showed that 81% of these cells possessed a nuclear label.

Sections from H^3 -uridine injected animals sacrificed after 48 hours exposure were quite similar and consistent with those recovered after one day.

The same pattern extended to single growing oocytes after 72 hours exposure to uridine. The nuclear labeling of the smallest single oocytes was identical to that seen in the larger oocyte packets. The concentration appeared more intense, particularly around the nucleolus, as the oocytes grew larger and reached a peak in the mature oocytes, while the cytoplasmic uptake was relatively light. This distribution is just opposite to that seen in the young developing oocyte packets which are compared in Figure 17.

Enzyme treatment. Pretreatment of the egg sections with RNase eliminated both the cytoplasmic and nucleolar label after a two-hour pulse with H^3 -uridine. While the nuclear label was reduced, scattered single oocytes and packets still possessed a moderately heavy label (Fig. 18). Thus it appeared that a large part of the nuclear label was not susceptible to RNase digestion.

Essentially similar results were obtained in eggs harvested after 8 hours. Most cells, in both packets and single oocyte stages, continued to show a moderate concentration of nuclear grains. Scattered single oocytes showed a heavier label over the nucleus and nucleolus that extended into the cytoplasm as well. Sections of the same eggs were pretreated with RNase before dipping into emulsion. Again this resulted in a complete loss of cytoplasmic and nucleolar labeling as well as the nuclear label from many of the single oocytes. In some of the large single oocytes, however, a heavy nuclear label still persisted after RNase treatment.

Consequently, similar sections were pretreated with DNase but this action did not remove any of the nuclear, nucleolar or cytoplasmic labeling.

Oocytes were harvested and animals were sacrificed at an extended interval of 6 days after injection of tritiated uridine. By the sixth day the intensity of the label had increased considerably. As indicated previously the ovarian oocytes were evenly and densely covered by fine grains. In the coelom, heavy labeling was again seen over the cytoplasm and nuclei of the smaller single oocytes. In larger oocytes the label appeared more concentrated in the karyoplasm of the nucleus and the nuclear membrane, and closely adherent to the nucleolar perimeter. This concentration became more accentuated in the oocytes approaching maturation size. In addition, the cytoplasm of the mature oocytes was evenly and densely covered by fine grains as shown in Figure 19. The outstanding difference noted for single oocytes in all stages of growth was an increase in grains over the cytoplasm. The amount of uptake became so intense that it equalled the original nuclear concentration.

DISCUSSION AND CONCLUSIONS

The gametes of *Pectinaria* generally were retained in the coelomic cavity over the long period of growth and maturation; only during the latter part of August were gametes found within the lumen of the nephromixia. This suggested that either the ciliated funnels of the nephromixia rejected the gametes until the spawning period or that some mechanism triggered their entrance into the nephromixia. Howie (1961a) observed that changes in the male coelomic gametes of *Arenicola marina* allowed them to be taken up by the nephromixia and subsequently shed. Further experiments with females (Howie, 1961b) indicated that the gonoducts would accept only mature oocytes and that non-spawning individuals could be induced to shed with injections from spawning females.

The proposal is made, based upon the evidence presented here and the observations in *Arenicola* by Howie, that the mature gametes in *Pectinaria* are selected or allowed to enter the nephromixia just prior to spawning. Full maturation of these oocytes, accomplished while the oocytes are still within the nephromixia, is accompanied by changes in the nucleus leading to G.V. breakdown and arrestment in metaphase I (Fig. 5). It is important to note that upon artificial stimulation or release of the oocytes from the coelomic cavity, the same stage in meiosis is reached. More than likely, the low fertility of these artificially shed eggs (Austin, 1963; Tweedell, 1962) is due to premature stimulation (*i.e.*, G.V. breakdown) of these eggs prior to their complete maturation.

During the growth or vegetative period of oocyte development in *Pectinaria* there is a lack of thymidine incorporation into the germinal vesicle, a condition that extends at least to the metaphase of the first maturation division. This response is the same as that in oocytes of the sea urchin (Ficq *et al.*, 1963b), the starfish (Geuskens, 1963), the sand dollar (Simmel and Karnovsky, 1961) and the cricket (Favard-Séréno and Durand, 1963b).

Nuclear uptake of tritiated thymidine and DNA synthesis is apparently restricted to the early generative stage prior to the growth of the germinal vesicle which occurs in the ovary of *Pectinaria*.

The cytoplasm of the early oocyte packets of *Pectinaria* does appear to utilize H^3 -thymidine, presumably in the synthesis of cytoplasmic DNA. Furthermore, the labeled material is probably retained in the cytoplasm of the developing oocyte to maturity. This may account for the extra DNA content that has been reported for many eggs (Grant, 1965). In *Ilyanassa*, Collier and McCann-Collier (1962) estimated the amount of DNA of the ovarian eggs to be 32 times that predicted for chromosomal DNA.

Generally, attempts to demonstrate cytoplasmic DNA in frog oocytes by autoradiography have been unsuccessful although Ficq (1961a) did find that the cytoplasm of non-dividing follicle cells became labeled but not the oocyte cytoplasm. In the cricket oocyte, however, the uptake of thymidine by follicular cell cytoplasm was followed by the transfer of radioactive DNA into the ooplasm (Favard-Séréno and Durand, 1963b).

The heavy concentration of radioactive label over the cytoplasm of mature oocytes after a prolonged exposure to H^3 -thymidine might be attributed to the accumulation of breakdown products of the original nucleoside. However, the differential uptake of the radioactive thymidine by the cells renders this unlikely. Alternatively, the buildup of cytoplasmic label could result from a migration of previously labeled nuclear DNA after the packets had reached maturity. Just as possible, the collection of cytoplasmic granules could result from cytoplasmic uptake as seen in the early oocyte packet phase with the subsequent fragmentation and development into single mature coelomic oocytes. This seems likely since the cytoplasmic label in the single oocytes appeared only after progressively longer exposures to the H^3 -thymidine, finally occurring over 17% of the largest oocytes after 21 days.

The rapid uptake of H^3 -uridine into the nuclear sap and particularly the nucleolus of the growing oocytes of *Pectinaria* and the ultimate transfer of the label to the cytoplasm of older oocytes strongly indicated that much of the uridine was used in the production of nuclear RNA. It also supports the contention that most of the cellular RNA used for cytoplasmic growth originates in the nucleus (Prescott, 1960).

Zalokar (1959, 1960) found that after one- to 4-minute exposures of *Neurospora* hyphae to H^3 -uridine, followed by centrifugation, only the RNA in the stratified nuclei became labeled. He indicated that at least 99% of the cellular RNA originated in the nucleus. After increased exposures, almost all of the label was found in the cytoplasm.

Ficq (1961a) also found, in autoradiographic studies, that all RNA synthesis in amphibian oocytes is nuclear.

During the diplotene phase of the amphibian oocyte, RNA is actively synthesized along the chromosome. In an autoradiographic study of the newt oocyte, Gall and Callan (1962) found a progressive incorporation of H^3 -uridine which moved sequentially along the loops of the lampbrush chromosomes. Microelectrophoretic measurements of the base composition for chromosomal RNA of *Triturus* oocytes indicated low guanine and cytosine, similar to DNA, and suggested m-RNA (Edstrom and Gall, 1963).

Quite similar results to the present study were found in an investigation of free oocytes from the sea urchin *Lytechinus pictus* by Piatigorsky, Ozaki and Tyler

(1966). There was an appreciable amount of C^{14} incorporation in RNA of the oocytes after 15- to 60-minute exposures, measured both quantitatively and by autoradiography. Labeling was found in the germinal vesicle and quite heavily in the nucleolus where uptake was almost entirely blocked by actinomycin D. In mature eggs very little, if any, incorporation of C^{14} -uridine could be measured and none was detected by labeling.

It is likely that much of the labeled nuclear RNA in *Pectinaria* oocytes is m-RNA. Edstrom *et al.* (1961) have shown that the base composition of nuclear RNA in the starfish oocyte resembles DNA and is probably m-RNA. Likewise, Ficq (1961a, 1961b, 1964; Ficq *et al.*, 1963) has long proposed that the uptake of H^3 -uridine and H^3 -cytidine in the nucleoplasm of various oocytes is due to a high molecular weight, metabolically active RNA, in close contact with DNA, that could be messenger RNA.

More direct evidence of messenger, *i.e.*, chromosomal, RNA was obtained by Sirlin, Jacob and Kato (1962). When a specific block, such as thioacetamide, is applied to the nuclei of chironomid larvae, the nucleolar RNA is inhibited from uptake of H^3 -uridine and free m-RNA appears instead. This labeled m-RNA was observed directly in the nucleus and followed to the cytoplasm.

Further indication that H^3 -uridine was incorporated into nuclear and nucleolar RNA in the present study resulted when the nucleolar label in all oocytes and part of the nuclear label in most oocytes was removed when they were submitted to RNase prior to autoradiography. Similar autoradiographs of H^3 -uridine uptake into the nuclear sap and nucleoli of oocytes have been shown in the cricket (Favard-Séréno and Durand, 1963a), the sea urchin (Ficq *et al.*, 1963), the starfish Geuskens, 1963) and the toad (Ficq, 1964).

The failure of RNase to remove the entire nuclear label from *some* of the larger, single oocytes of *Pectinaria* may be significant since H^3 -uridine may act as a precursor for DNA. During vitellogenesis in the cricket oocyte, uridine is incorporated into DNA in the same manner as the uptake of thymidine (Favard-Séréno and Durand, 1963b). Beliaevsky and Tencer (1960) noted that uridine is selectively taken into DNA of amphibian embryos at gastrulation. Collier (1963a, 1965) also found C^{14} -uridine acts as a major precursor in the synthesis of DNA in the *Ilyanassa* embryo.

The same retention of nuclear label was found in H^3 -uridine-labeled oocytes of *Pectinaria* after pre-treatment with DNase. If some of the H^3 -uridine is converted to DNA, the marked compound must be tied up, possibly as a RNA-DNA hybrid. This awaits further investigation.

The tremendous increase in size, and particularly the budding activity of the nucleoli, during oocyte development and growth in *Pectinaria* also suggest an active production of RNA. Previous results show that both the epinucleolar and intranucleolar buds fluoresce bright red with acridine orange (Tweedell, 1962), another indication of RNA. Nucleolar extrusion and emission of nucleolar products into the cytoplasm is well documented for other oocytes (Raven, 1961). Consequently, the heavy labeling of the nucleolar buds in the primary oocyte of *Pectinaria* supported this viewpoint.

The evidence for nucleolar incorporation of RNA precursors into developing oocytes has been well documented (Ficq, 1961, 1962, 1964; Ficq *et al.*, 1963;

Favard-Séréno and Durand, 1963a; Ozban *et al.*, 1964). Furthermore, Brown and Gurdon (1964) found that there is a total absence of ribosomal RNA synthesis in embryos of an anucleolate mutant of *Xenopus laevis*, even though other RNA was being synthesized.

Yet the relationship between the exact site of nucleolar RNA synthesis, the chromosome and the organizer DNA has not been too clear.

Zalokar (1962) believes the nucleoli are only temporary storage places for newly formed RNA. Following incorporation of H^3 -uridine into RNA of oocyte nuclei of *Blatella germanica*, he found that the RNA was detected in the chromosomal region peripheral to the nucleolus. The nucleoli remained unlabeled but chromosomes were labeled when moderate amounts of actinomycin were applied.

In experiments on the salivary gland nuclei of chironomid larvae (*Smittia* sp.) Sirlin *et al.* (1962) pulse-labeled the cells with H^3 -uridine and pretreated with TRB, a general RNA inhibitor. The nucleoli were heavily labeled while the nucleolar organizer remained unlabeled. They presented evidence for an extrinsic nucleolar RNA (chromosomal RNA) and an intrinsic nucleolar RNA, and proposed that the chromosomal RNA, not organizer DNA, primed the intrinsic nucleolar RNA.

On the other hand, when isolated amphibian oocytes were exposed *in vitro* to H^3 -uridine, H^3 -cytidine, incorporation into the lampbrush chromosomes took place (Izawa *et al.*, 1963). However, prior application of actinomycin D blocked uptake in both the chromosomal loops and the nucleolus. From this they concluded that RNA synthesis in both areas is DNA-dependent.

The type of RNA synthesized and the sequential movement of RNA within the nucleus are still very controversial.

One model for the origin of RNA synthesis (Perry, 1965) proposes that the nucleolus accounts for roughly two-thirds of the cytoplasmic RNA (r-RNA species) while extranucleolar synthesis accounts for m-RNA and t-RNA.

It is germane to examine which kinds of RNA are involved in the labeling of the oocyte nucleus and particularly the nucleolus. All three types of RNA (t-RNA, r-RNA and m-RNA) have been recognized in the nucleoli of oocytes and other cells (Sirlin *et al.*, 1963). Direct evidence for two RNA types in the nucleolus was obtained from fractionation of the nucleoli of pea seedlings (Birnstiel and Chipchase, 1963), where it was found that 56% of the extractable RNA is t-RNA (4s), the remainder consisting of ribosomal species.

At least two types of nuclear RNA, which varied in their solubility and specific activity, have been found in the starfish oocyte nucleus and nucleolus (Vincent, 1954, 1957). Vincent and Baltus (1960) later found that the oocyte nucleolus of the starfish binds C^{14} -leucine to an "activation" RNA that is attached to a 4.5s protein, presumably used in the synthesis of cellular proteins.

Possible supporting evidence of nucleolar production of t-RNA was presented by Fleissner and Borek (1962) in the transmethylation of (C^{14} methyl) methionine to ribonucleic acid. Sirlin, Jacob and Tandler (1963) were able to show by autoradiography preferential uptake of (methyl C^{14}) methionine into the nucleolus of the salivary glands of a chironomid. When puromycin was applied to block the incorporation of methionine into the protein of the chromosomes and cytoplasm, the latter showed little or no uptake. The remaining nucleolar label was entirely

removed by RNase. This was interpreted as the transfer of the methyl group to t-RNA of the nucleolus.

The same formation of nucleolar RNA from (methyl C^{14}) methionine has been obtained in developing oocytes of the toad (Ozban, Tandler and Sirlin, 1964). The increase of incorporation into RNase-sensitive nucleolar material was proportional to oocyte growth, *e.g.*, cytoplasmic growth. In older larger oocytes, almost all nucleolar label derived in the presence of puromycin was RNase-sensitive.

In contrast, Ficq (1961) found t-RNA was formed in the cytoplasm of amphibian oocytes from labeled cytidine. More recently, Ficq (1966) combined methyl- C^{14} -methionine with enzymatic digestion by ribonuclease on the oocytes of newts and salamanders. The methylating activity was reported in the cytoplasm as well as the nucleolus; she did not find the localization of t-RNA to be preferentially nucleolar.

Birnstiel, Fleissner and Borek (1963) have reported that RNA methylases are concentrated in the nucleoli of pea nuclei where the enzymes are believed to alter t-RNA by the incorporation of methyl groups into the component bases.

Davidson, Allfrey and Mirsky (1964) concluded that during the lampbrush stage of oogenesis the oocytes of *Xenopus laevis* produce huge quantities of ribosomal RNA. After injection of H^3 -uridine, they found that over 90% of the RNA recovered was of the ribosomal type. In the amphibian most of this RNA is produced in early oocytes rather than in ovulated eggs. After isolation of RNA from immature (ovarian) oocytes of *Xenopus* with P^{32} the immature oocytes produced an abundance of two ribosomal species whereas no labeled ribosomal RNA was found in ovulated eggs (Brown and Littna, 1964).

The nucleolar and cytoplasmic RNA in the starfish oocyte have very similar base ratios (Edstrom *et al.*, 1961), suggesting that ribosomal RNA is a result of this nucleolar activity. In oocytes of *Triturus*, Edstrom and Gall (1963) similarly reported there is an overall resemblance between nucleolar and cytoplasmic RNA. Both are rich in guanine-cytosine compared to relatively low G-C content of chromosomal RNA. Furthermore, Ficq (1964) finds that H^3 -cytidine, H^3 -5-methylcytosine and H^3 -uridine are preferentially incorporated into RNA of the oocyte nucleolus of the sea urchin and toad; this uptake is completely blocked by actinomycin D.

Radiography and extraction of RNA from the sea urchin oocytes (Gross *et al.*, 1965) confirm the contention that H^3 -uridine is incorporated into several types of RNA during oocyte development. One week after injection of H^3 -uridine into *Arbacia punctulata* the whole ovarian region became radioactive. The smaller ovarian oocytes were heavily labeled in both nuclei and cytoplasm while the larger oocytes had radioactivity primarily in the cytoplasm. The number of ootids with a cytoplasmic label varied from $\frac{3}{4}$ of the cells intermediate in the acinus of the ovary to only a few mature ootids from the central lumen. A few were heavily labeled in the cytoplasm and nucleus. Some of the central ootids contained labeled nuclei although "rarely with significant radioactivity." Apparently these were labeled during late maturation stages.

Subsequent extraction of RNA from these central ootids yielded three major RNA components. Two were 28S and 18S ribosomal species; the third was 4S

material, some of which was t-RNA. Another fraction was presumed to be m-RNA.

The relatively light cytoplasmic uptake of H^3 -uridine into oocytes of *Pectinaria* after short pulses or moderate exposures indicated that little uridine was taken directly into cytoplasmic RNA. There was, however, a slow increase in cytoplasmic label after two days that increased dramatically after a six-day exposure, which might represent a latent uptake of uridine into ribosomal RNA. However, if nucleolar RNA and ribosomal RNA are related, the cytoplasmic buildup could be due to a migration of nuclear RNA into the cytoplasm. Indeed, the nuclear and nucleolar label does diminish as the cytoplasmic activity increases, a situation similar to that in the cricket oocytes (Favard-Séréno and Durand, 1963a) and in the starfish oocytes (Edstrom *et al.*, 1961).

We can conclude, then, that H^3 -uridine is incorporated into several types of RNA, both nuclear and nucleolar in origin, during oocyte development. Presumably the nuclear label is due to m-RNA and ribosomal RNA does appear to be derived directly from nucleolar RNA.

SUMMARY

1. The development and growth of the primary oocytes in the coelomic fluid are described. Oocytes progress from a packet stage to a single oocyte stage accompanied by vegetative growth of the germinal vesicle. Evidence presented indicates that the germinal vesicle breaks down and the oocyte reaches the first maturation division prior to shedding under natural conditions.

2. Nuclear uptake of H^3 -thymidine is confined to the ovarian cells following the last oogonal division in the premeiotic phase. There is evidence that free coelomic oocyte packets incorporate H^3 -thymidine directly into the cytoplasm. The cytoplasmic label is removed by treatment with DNase.

3. Short pulses of H^3 -uridine are taken up diffusely by the ovarian oocytes, while small packets and single oocytes incorporate H^3 -uridine primarily in the nucleus and in the nucleolus. Extended exposure from 4 to 48 hours indicates that some of the nuclear uridine moves into the cytoplasm in the packet oocytes. Individual oocytes show strong nuclear labeling up to 72 hours after injection. Particularly long exposures of 6 days indicate that the uridine accumulates in the cytoplasm in the largest oocytes.

4. Treatment with RNase removes most of the nuclear and cytoplasmic H^3 -uridine label. Some of the nuclear label is resistant and is not removed by RNase or DNase.

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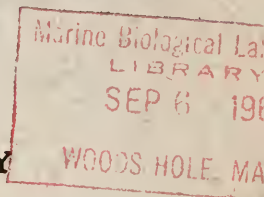
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THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY



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AUGUST, 1966

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

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THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, \$3.75. Subscription per volume (three issues), \$9.00, (this is \$18.00 per year for six issues).

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